【Name of Document】 APPLICATION FOR PATENT

【Identification Number】 C1-A0320

【Filing Date】 December 12, 2003

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　　【Registration Number】　　041092

　　【Amount】　　　　　　　21000 yen

【List of Attached Documents】

　　【Name of Document】　Claims　　　　　1

　　【Name of Document】　Specification　　1

　　【Name of Document】　Drawings　　　　1

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【General Power of Attorney Number】　0216136

[Document Name] Claims

[Claim 1]

An antibody comprising a single-chain polypeptide having binding activity against TPO receptor (Mpl), wherein said antibody comprises two heavy chain variable regions and two light chain variable regions.

[Claim 2]

The antibody of claim 1, wherein the two heavy chain variable regions and the two light chain variable regions are arranged in the order of heavy chain variable region, light chain variable region, heavy chain variable region, and light chain variable region from the N terminus of the single-chain polypeptide.

[Claim 3]

The antibody of claim 1 or 2, wherein the two heavy chain variable regions and the two light chain variable regions are linked by linkers.

[Claim 4]

The antibody of claim 3, wherein the linkers comprise 15 amino acids.

[Claim 5]

A chimeric antibody that binds to Mpl.

[Claim 6]

The antibody of claim 5, which is a humanized antibody.

[Claim 7]

The antibody of claim 5 or 6, which is a minibody.

[Claim 8]

An antibody that binds to soluble Mpl.

[Claim 9]

An antibody that binds to human Mpl and monkey Mpl.

[Claim 10]

An antibody having agonistic activity against human Mpl and monkey Mpl.

[Claim 11]

An antibody whose binding activity to soluble Mpl is $KD = 10^{-6}$ M or lower.

[Claim 12]

An antibody whose binding activity to soluble Mpl is $KD = 10^{-7}$ M or lower.

[Claim 13]

An antibody whose binding activity to soluble Mpl is $KD = 10^{-8}$ M or lower.

[Claim 14]

An antibody whose TPO agonistic activity is $EC50 = 100$ nM or lower.

[Claim 15]

An antibody whose TPO agonistic activity is EC50 = 30 nM or lower.

[Claim 16]

An antibody whose TPO agonistic activity is EC50 = 10 nM or lower.

[Claim 17]

An antibody which comprises a heavy chain variable region, wherein said heavy chain variable regions comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

[1] SEQ ID NOs: 3, 4, and 5
[2] SEQ ID NOs: 6, 7, and 8
[3] SEQ ID NOs: 9, 10, and 11
[4] SEQ ID NOs: 12, 13, and 14
[5] SEQ ID NOs: 15, 16, and 17
[6] SEQ ID NOs: 18, 19, and 20
[7] SEQ ID NOs: 21, 22, and 23
[8] SEQ ID NOs: 24, 25, and 26
[9] SEQ ID NOs: 27, 28, and 29
[10] SEQ ID NOs: 30, 31, and 32
[11] SEQ ID NOs: 33, 34, and 35
[12] SEQ ID NOs: 36, 37, and 38
[13] SEQ ID NOs: 39, 40, and 41
[14] SEQ ID NOs: 42, 43, and 44
[15] SEQ ID NOs: 45, 46, and 47
[16] SEQ ID NOs: 48, 49, and 50
[17] SEQ ID NOs: 51, 52, and 53
[18] SEQ ID NOs: 54, 55, and 56
[19] SEQ ID NOs: 57, 58, and 59

[Claim 18]

An antibody which comprises a light chain variable region, wherein said light chain variable region comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

[1] SEQ ID NOs: 60, 61, and 62
[2] SEQ ID NOs: 63, 64, and 65
[3] SEQ ID NOs: 66, 67, and 68
[4] SEQ ID NOs: 69, 70, and 71
[5] SEQ ID NOs: 72, 73, and 74
[6] SEQ ID NOs: 75, 76, and 77

[7] SEQ ID NOs: 78, 79, and 80

[8] SEQ ID NOs: 81, 82, and 83

[9] SEQ ID NOs: 84, 85, and 86

[10] SEQ ID NOs: 87, 88, and 89

5         [11] SEQ ID NOs: 90, 91, and 92

[12] SEQ ID NOs: 93, 94, and 95

[13] SEQ ID NOs: 96, 97, and 98

[14] SEQ ID NOs: 99, 100, and 101

[15] SEQ ID NOs: 102, 103, and 104

10        [16] SEQ ID NOs: 105, 106, and 107

[17] SEQ ID NOs: 108, 109, and 110

[18] SEQ ID NOs: 111, 112, and 113

[19] SEQ ID NOs: 114, 115, and 116.

[Claim 19]

15        An antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 3, 4, and 5, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting

20    of SEQ ID NOs: 60, 61, and 62;

[2] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 6, 7, and 8, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 63, 64, and 65;

25        [3] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 9, 10, and 11, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 66, 67, and 68;

[4] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising

30    the amino acid sequences consisting of SEQ ID NOs: 12, 13, and 14, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 69, 70, and 71;

[5] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 15, 16, and 17, and a light chain variable

35    region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 72, 73, and 74;

[6] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 18, 19, and 20, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 75, 76, and 77;

[7] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 21, 22, and 23, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 78, 79, and 80;

[8] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 24, 25, and 26, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 81, 82, and 83;

[9] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 27, 28, and 29, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 84, 85, and 86;

[10] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 30, 31, and 32, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 87, 88, and 89;

[11] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 33, 34, and 35, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 90, 91, and 92;

[12] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 36, 37, and 38, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 93, 94, and 95;

[13] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 39, 40, and 41, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 96, 97, and 98;

[14] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 42, 43, and 44, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 99, 100, and 101;

[15] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 45, 46, and 47, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 102, 103, and 104;

5          [16] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 48, 49, and 50, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 105, 106, and 107;

          [17] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising

10       the amino acid sequences consisting of SEQ ID NOs: 51, 52, and 53, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 108, 109, and 110;

          [18] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 54, 55, and 56, and a light chain variable

15       region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 111, 112, and 113;

          [19] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 57, 58, and 59, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting

20       of SEQ ID NOs: 114, 115, and 116.

[Claim 20]

          An antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118.

[Claim 21]

25       An antibody that comprises a light chain variable region comprising the amino acid sequence of SEQ ID NO: 120.

[Claim 22]

          An antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118 and a light chain variable region comprising the amino acid

30       sequence of SEQ ID NO: 120.

[Claim 23]

          An antibody comprising the amino acid sequence of SEQ ID NO: 122.

[Claim 24]

          An antibody comprises the amino acid sequence of SEQ ID NO: 2.

35       [Claim 25]

          An antibody having an activity equivalent to that of an antibody of any one of claims 17

to 24, wherein said antibody comprises the amino acid sequence set forth in any one of claims 17 to 24, in which one or more amino acids have been substituted, deleted, added and/or inserted.
[Claim 26]

An antibody that recognizes an epitope recognized by an antibody of any one of claims 17 to 25.
[Claim 27]

An antibody that recognizes the region of amino acids 26 to 274 of human Mpl.
[Claim 28]

An antibody of any one of claims 1 to 27, which has TPO agonistic activity.
[Claim 29]

A polynucleotide encoding an antibody of any one of claims 1 to 28.
[Claim 30]

A polynucleotide hybridizing to the polynucleotide of claim 29 under stringent conditions, wherein said polynucleotide encodes an antibody having activity equivalent to that of an antibody of any one of claims 1 to 28.
[Claim 31]

A vector comprising the polynucleotide of claim 29 or 30.
[Claim 32]

A host cell that carries the polynucleotide of claim 29 or 30, or the vector of claim 31.
[Claim 33]

A pharmaceutical composition comprising an antibody of any one of claims 1 to 28.

[Document Name] Specification

[Title of the Invention] ANTI-MPL ANTIBODIES

[Technical Field]

[0001]

The present invention relates to anti-Mpl antibodies.

[Background of the Invention]

[0002]

Thrombopoietin (TPO) is a factor that enhances the differentiation and maturation of megakaryocytes (platelet precursor cells) from hemopoietic stem cells into platelets.   TPO also functions as a cytokine with an important role in the regulation of platelet number.   TPO is converted into its active form through the cleavage of a TPO precursor comprising 353 amino acids.

Mpl is a TPO receptor, and human Mpl molecules are known to exist in two forms comprising 572 and 635 amino acids.   The human Mpl gene sequence has already been analyzed (see Non-patent Document 1 and GenBank accession No. NM_005373).

Most cytokine receptors dimerize upon ligand binding, and transduce signals into cells. It has been reported that TPO similarly binds to its own specific receptor MPL, which leads to dimerization of the receptor, thereby transducing signals into cells and exerting physiological effects (see Non-patent Document 2).

Antibodies exhibiting agonistic activity have been reported among those antibodies that bind to receptors having the above features.

For example, an antibody against the erythropoietin (EPO) receptor has been reported to substitute for erythropoietin function.   The monovalent form (Fab) of the antibody is capable of binding to the EPO receptor but is unable to transduce signals.   Thus, dimerization of the erythropoietin receptor via bivalent binding is assumed to be essential for signal transduction (see Non-patent Document 3).

Antibodies that bind to Mpl and exhibit TPO agonistic activity have also been reported (see Non-patent Documents 4 and 5).   This suggests that receptor dimerization is induced upon binding of a bivalent antibody with regards to MPL as well.

Meanwhile, a single-chain antibody (scFv) has been reported to exhibit TPO agonistic activity (see Patent Document 1).   However, it has been revealed that, the underlying mechanism of scFv exhibiting TPO agonistic activity is that a part of scFv dimerizes (diabody) and this diabody becomes the actual active unit (see Patent Documents 2 to 4).

[0003]

[Patent Document 1] US Patent No. 6342220

[Patent Document 2] WO 01/79494

[Patent Document 3] WO 02/33072

[Patent Document 4] WO 02/33073

[Non-patent Document 1] Palacios *et al.*, 1985, Cell, 41, 727-734

[Non-patent Document 2] Souyri *et al.*, 1990, Cell, Vol.63, 1137-1147

[Non-patent Document 3]Elliott, S. *et al.*, 1996, J. Biol. Chem., 271(40), 24691-24697

[Non-patent Document 4] Abe *et al.*, 1998, Immunol. Lett., 61, 73-78

[Non-patent Document 5] Bijia Deng *et al.*, 1998, Blood, 92, 1981-1988

[Disclosure of the Invention]

[Problems to be Solved by the Invention]

[0004]

The present invention was achieved in view of the above circumstances.　An objective of the present invention is to provide novel anti-Mpl antibodies having TPO agonistic activity.

[Means for Solving the Problems]

[0005]

The present inventors performed exhaustive research to solve the above objective.　The present inventors prepared and purified anti-human Mpl antibody VB22B, and established a single-chain antibody expression system using genetic engineering techniques.　Specifically, the variable region of anti-human Mpl antibody was first cloned, and a diabody expression vector pCXND3-VB22B db for the anti-human Mpl antibody was prepared.　This pCXND3-VB22B db vector was then used to generate an expression vector pCXND3-VB22B sc(Fv)$_2$ for anti-human Mpl antibody sc(Fv)$_2$.　Anti-human Mpl sc(Fv)$_2$ was transiently expressed in CHO-DG44 cells using the expression vector pCXND3-VB22B sc(Fv)$_2$, and then purified from the culture supernatant.　In control experiments, VB22B diabody was transiently expressed in COS7 cells using the above pCXND3-VB22B db vector, and then purified from the culture supernatant.

[0006]

In addition, VB22B diabody and VB22B sc(Fv)$_2$ were evaluated for their TPO-like agonistic activities.　The results showed that VB22B diabody and VB22B sc(Fv)$_2$ exhibit higher agonistic activities compared to VB22B IgG, and thus activities equivalent to or higher than that of the natural ligand, human TPO.

[0007]

More specifically, the present invention relates to:

(1) an antibody comprising a single-chain polypeptide having binding activity against TPO receptor (Mpl), wherein said antibody comprises two heavy chain variable regions and two light chain variable regions;

(2) the antibody of (1), wherein the two heavy chain variable regions and the two light chain variable regions are arranged in the order of heavy chain variable region, light chain variable region, heavy chain variable region, and light chain variable region from the N terminus of the single-chain polypeptide;

(3) the antibody of (1) or (2), wherein the two heavy chain variable regions and the two light chain variable regions are linked by linkers;

(4) the antibody of (3), wherein the linkers comprise 15 amino acids;

(5) a chimeric antibody that binds to Mpl;

(6) the antibody of (5), which is a humanized antibody;

(7) the antibody of (5) or (6), which is a minibody;

(8) an antibody that binds to soluble Mpl;

(9) an antibody that binds to human Mpl and monkey Mpl;

(10) an antibody having agonistic activity against human Mpl and monkey Mpl;

(11) an antibody whose binding activity to soluble Mpl is $KD = 10^{-6}$ M or lower;

(12) an antibody whose binding activity to soluble Mpl is $KD = 10^{-7}$ M or lower;

(13) an antibody whose binding activity to soluble Mpl is $KD = 10^{-8}$ M or lower;

(14) an antibody whose TPO agonistic activity is EC50 = 100 nM or lower;

(15) an antibody whose TPO agonistic activity is EC50 = 30 nM or lower;

(16) an antibody whose TPO agonistic activity is EC50 = 10 nM or lower;

(17) an antibody which comprises a heavy chain variable region, wherein said heavy chain variable regions comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

[1] SEQ ID NOs: 3, 4, and 5

[2] SEQ ID NOs: 6, 7, and 8

[3] SEQ ID NOs: 9, 10, and 11

[4] SEQ ID NOs: 12, 13, and 14

[5] SEQ ID NOs: 15, 16, and 17

[6] SEQ ID NOs: 18, 19, and 20

[7] SEQ ID NOs: 21, 22, and 23

[8] SEQ ID NOs: 24, 25, and 26

[9] SEQ ID NOs: 27, 28, and 29

[10] SEQ ID NOs: 30, 31, and 32

[11] SEQ ID NOs: 33, 34, and 35

[12] SEQ ID NOs: 36, 37, and 38

[13] SEQ ID NOs: 39, 40, and 41

[14] SEQ ID NOs: 42, 43, and 44

[15] SEQ ID NOs: 45, 46, and 47

[16] SEQ ID NOs: 48, 49, and 50

[17] SEQ ID NOs: 51, 52, and 53

[18] SEQ ID NOs: 54, 55, and 56

[19] SEQ ID NOs: 57, 58, and 59;

(18) an antibody which comprises a light chain variable region, wherein said light chain variable region comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

[1] SEQ ID NOs: 60, 61, and 62

[2] SEQ ID NOs: 63, 64, and 65

[3] SEQ ID NOs: 66, 67, and 68

[4] SEQ ID NOs: 69, 70, and 71

[5] SEQ ID NOs: 72, 73, and 74

[6] SEQ ID NOs: 75, 76, and 77

[7] SEQ ID NOs: 78, 79, and 80

[8] SEQ ID NOs: 81, 82, and 83

[9] SEQ ID NOs: 84, 85, and 86

[10] SEQ ID NOs: 87, 88, and 89

[11] SEQ ID NOs: 90, 91, and 92

[12] SEQ ID NOs: 93, 94, and 95

[13] SEQ ID NOs: 96, 97, and 98

[14] SEQ ID NOs: 99, 100, and 101

[15] SEQ ID NOs: 102, 103, and 104

[16] SEQ ID NOs: 105, 106, and 107

[17] SEQ ID NOs: 108, 109, and 110

[18] SEQ ID NOs: 111, 112, and 113

[19] SEQ ID NOs: 114, 115, and 116;

(19) an antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 3, 4, and 5, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 60, 61, and 62;

[2] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 6, 7, and 8, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting

of SEQ ID NOs: 63, 64, and 65;

[3] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 9, 10, and 11, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 66, 67, and 68;

[4] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 12, 13, and 14, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 69, 70, and 71;

[5] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 15, 16, and 17, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 72, 73, and 74;

[6] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 18, 19, and 20, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 75, 76, and 77;

[7] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 21, 22, and 23, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 78, 79, and 80;

[8] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 24, 25, and 26, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 81, 82, and 83;

[9] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 27, 28, and 29, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 84, 85, and 86;

[10] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 30, 31, and 32, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 87, 88, and 89;

[11] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 33, 34, and 35, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting

of SEQ ID NOs: 90, 91, and 92;

[12] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 36, 37, and 38, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 93, 94, and 95;

[13] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 39, 40, and 41, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 96, 97, and 98;

[14] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 42, 43, and 44, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 99, 100, and 101;

[15] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 45, 46, and 47, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 102, 103, and 104;

[16] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 48, 49, and 50, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 105, 106, and 107;

[17] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 51, 52, and 53, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 108, 109, and 110;

[18] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 54, 55, and 56, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 111, 112, and 113;

[19] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 57, 58, and 59, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 114, 115, and 116;

(20) an antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118;

(21) an antibody that comprises a light chain variable region comprising the amino acid

sequence of SEQ ID NO: 120;

(22) an antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 120;

(23) an antibody comprising the amino acid sequence of SEQ ID NO: 122;

(24) an antibody comprises the amino acid sequence of SEQ ID NO: 2;

(25) an antibody having an activity equivalent to that of an antibody of any one of (17) to (24), wherein said antibody comprises the amino acid sequence set forth in any one of (17) to (24), in which one or more amino acids have been substituted, deleted, added and/or inserted;

(26) an antibody that recognizes an epitope recognized by an antibody of any one of (17) to (25);

(27) an antibody that recognizes the region of amino acids 26 to 274 of human Mpl;

(28) an antibody of any one of (1) to (27), which has TPO agonistic activity;

(29) a polynucleotide encoding an antibody of any one of (1) to (28);

(30) a polynucleotide hybridizing to the polynucleotide of (29) under stringent conditions, wherein said polynucleotide encodes an antibody having activity equivalent to that of an antibody of any one of (1) to (28);

(31) a vector comprising the polynucleotide of (29) or (30);

(32) a host cell that carries the polynucleotide of (29) or (30), or the vector of (31); and

(33) a pharmaceutical composition comprising an antibody of any one of (1) to (28).

[Effects of the Invention]

[0008]

Recombinant human TPOs have been tested as therapeutic agents for chemotherapy-induced thrombocytopenia in various clinical trials.   In these trials, a major problem that was reported was the production of anti-TPO antibody in TPO-treated patients (Junzhi Li, *et al.*, Blood (2001) 98: 3241-324; Saroj Vandhan-Raj, *et al.*, Ann. Intern. Med. (2000) 132: 364-368).   Specifically, production of neutralizing antibodies that inhibit endogenous TPO activity, and the resulting onset of thrombocytopenia, were reported. Administration of agonistic minibodies (low molecular weight antibodies) against anti-TPO receptor of the present invention does not induce the production of antibodies against endogenous TPO.   Furthermore, since antibody miniaturization results in increased specific activity and short half-life in blood, the effective concentration in blood can be easily regulated, presenting a further clinical advantage.   Accordingly, the present antibodies are expected to be therapeutic agents for thrombocytopenia which are more effective than naturally-occurring TPO or its agonistic antibodies.   Furthermore, since minibodies are not glycosylated, expression systems for expressing the recombinant proteins are not limited, and minibodies can be produced

in any expression system such as cell lines from mammals, yeast, insect cells, and *E. coli*.
Since binding specificity against mutant TPO receptor is different from that of TPO, minibodies
are expected to bind specific mutants and show agonistic activity against mutated TPO receptor
detected in the CAMT patients, who genetically have mutated TPO receptor and develop

5    thrombocytopenia.

[Best Mode for Carrying Out the Invention]

  [0009]

    The present invention provides antibodies that bind to the TPO receptor (Mpl).

    The antibodies of the present invention comprise all types of antibodies, including

10  antibodies with modified amino acid sequences, such as minibodies, humanized antibodies, and
chimeric antibodies; antibodies that have been modified by binding with other molecules (for
example, polymers such as polyethylene glycol); and antibodies whose sugar chains have been
modified.

    It is preferable that the antibodies of the present invention have agonistic activity against

15  Mpl.

  [0010]

    In a preferred embodiment, the antibodies of the present invention comprise, for
example, minibodies.

    The minibodies comprise antibody fragments lacking portions of the whole antibody

20  (for example, whole IgG).   The minibodies are not particularly limited as long as they have
binding activity to their antigens.   The minibodies of the present invention have markedly
higher activities compared to their corresponding whole antibodies.   There are no particular
limitations on the antibody fragments of the present invention as long as they are portions of the
whole antibody, and preferably contain heavy chain variable regions (VH) and/or light chain

25  variable regions (VL).   The amino acid sequences of VH or VL may contain substitutions,
deletions, additions and/or insertions.   Furthermore, the antibody fragment may also lack
portions of VH or/and VL, as long as it has binding ability to its antigen.   In addition, the
variable regions may be chimerized or humanized.   Such antibody fragments include, for
example, Fab, Fab', F(ab')$_2$, and Fv.   An example of a minibody includes Fab, Fab', F(ab')$_2$, Fv,

30  scFv (single-chain Fv), diabody, and sc(Fv)$_2$ (single-chain (Fv)$_2$).

  [0011]

    Herein, an "Fv" fragment is the smallest antibody fragment and contains a complete
antigen recognition site and a binding site.   The "Fv" fragment is a dimer (VH-VL dimer) in
which a single VH and a single VL are strongly linked by a non-covalent bond.   The three

35  complementarity-determining regions (CDRs) of each of the variable regions interact with each
other to form an antigen-binding site on the surface of the VH-VL dimer.   Six CDRs confer the

antigen-binding site of an antibody.   However, a single variable region (or a half of Fv containing only three CDRs specific to an antigen) alone is also capable of recognizing and binding an antigen although its affinity is lower than the affinity of the entire binding site.

[0012]

5        scFv contains the VH and VL regions of an antibody, and these regions exist on a single polypeptide chain.   Generally, an Fv polypeptide further contains a polypeptide linker between VH and VL, and therefore an scFv can form a structure required for antigen binding.   See, Pluckthun "The Pharmacology of Monoclonal Antibodies" Vol. 113 (Rosenburg and Moore eds. (Springer Verlag, New York, pp.269-315, 1994) for the review of scFv.   In the present invention,

10       linkers are not especially limited as long as they do not inhibit expression of antibody variable regions linked at both ends of the linkers.

[0013]

The term "diabody" refers to a bivalent antibody fragment constructed by gene fusion (Holliger P $et\ al.$, Proc. Natl. Acad. Sci. USA (1993) 90: 6444-6448; EP 404,097; WO 93/11161

15       and others).   Diabodies are dimers comprising two polypeptide chains, where each polypeptide chain comprises a VL and a VH connected with a linker short enough to prevent interaction of these two domains, for example, a linker of about five residues.   The VL and VH encoded on the same polypeptide chain will form a dimer because the linker between them is too short to form a single-chain variable region fragment.   As a result, the polypeptide chains form a dimer,

20       and thus the diabody has two antigen binding sites.

[0014]

sc(Fv)$_2$ is a single-chain minibody produced by linking two units of VH and two units of VL with linkers and such (Hudson $et\ al.$, J Immunol. Methods (1999) 231: 177-189).   sc(Fv)$_2$ exhibits a particularly high agonistic activity compared to the whole antibody and other

25       minibodies.   sc(Fv)$_2$ can be produced, for example, by linking two scFv molecules.

[0015]

In a preferable antibody, the two VH units and two VL units are arranged in the order of VH, VL, VH, and VL ([VH]-linker-[VL]-linker-[VH]-linker-[VL]) beginning from the N terminus of a single-chain polypeptide.

30       The order of the two VH units and two VL units is not limited to the above arrangement, and they may be arranged in any order.   Examples of the arrangements are listed below.
[VL]-linker-[VH]-linker-[VH]-linker-[VL]
[VH]-linker-[VL]-linker-[VL]-linker-[VH]
[VH]-linker-[VH]-linker-[VL]-linker-[VL]

35       [VL]-linker-[VL]-linker-[VH]-linker-[VH]
[VL]-linker-[VH]-linker-[VL]-linker-[VH]

[0016]

The linkers to be used for linking the variable regions of an antibody comprise arbitrary peptide linkers that can be introduced by genetic engineering, synthetic linkers, and linkers disclosed in, for example, Holliger, P. *et al.*, Protein Engineering (1996) 9 (3): 299-305.

Peptide linkers are preferred in the present invention.    There are no limitations as to the length of the peptide linkers.    The length can be selected accordingly by those skilled in the art depending on the purpose, and is typically 1-100 amino acids, preferably 3-50 amino acids, more preferably 5-30 amino acids, and even more preferably 12-18 amino acids (for example, 15 amino acids).

[0017]

For example, such peptide linkers include:

Ser

Gly Ser

Gly Gly Ser

Ser Gly Gly

Gly Gly Gly Ser

Ser Gly Gly Gly

Gly Gly Gly Gly Ser

Ser Gly Gly Gly Gly

Gly Gly Gly Gly Gly Ser

Ser Gly Gly Gly Gly Gly

Gly Gly Gly Gly Gly Gly Ser

Ser Gly Gly Gly Gly Gly Gly

(Gly Gly Gly Gly Ser)$_n$

(Ser Gly Gly Gly Gly)$_n$

where n is an integer of 1 or larger.    The lengths and sequences of peptide linkers can be selected accordingly by those skilled in the art depending on the purpose.

[0018]

In an embodiment of the present invention, a particularly preferable sc(Fv)$_2$ includes, for example, the sc(Fv)$_2$ below.

[VH]-peptide linker (15 amino acids)-[VL]-peptide linker (15 amino acids)-[VH]-peptide linker (15 amino acids)-[VL]

[0019]

Synthetic linkers (chemical crosslinking agents) include crosslinking agents routinely used to crosslink peptides, for example, N-hydroxy succinimide (NHS), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS$^3$), dithiobis(succinimidyl propionate) (DSP),

dithiobis(sulfosuccinimidyl propionate) (DTSSP), ethylene glycol bis(succinimidyl succinate) (EGS), ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST), bis[2-(succinimidoxycarbonyloxy)ethyl] sulfone (BSOCOES), and bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl] sulfone
5    (sulfo-BSOCOES).   These crosslinking agents are commercially available.

[0020]

In general, three linkers are required to link four antibody variable regions together. The linkers to be used may be of the same type or different types.   In the present invention, a preferable minibody is a diabody, even more preferably, an sc(Fv)$_2$.   Such a minibody can be
10   prepared by treating an antibody with an enzyme, for example, papain or pepsin, to generate antibody fragments, or by constructing DNAs encoding those antibody fragments and introducing them into expression vectors, followed by expression in an appropriate host cell (see, for example, Co, M. S. *et al.*, J. Immunol. (1994) 152: 2968-2976; Better, M. and Horwitz, A. H., Methods Enzymol. (1989) 178: 476-496; Pluckthun, A. and Skerra, A., Methods Enzymol.
15   (1989) 178: 497-515; Lamoyi, E., Methods Enzymol. (1986) 121: 652-663; Rousseaux, J. *et al.*, Methods Enzymol. (1986) 121: 663-669; Bird, R. E. and Walker, B. W., Trends Biotechnol. (1991) 9: 132-137).

An antibody having exceedingly high agonistic activity can be prepared by reducing the molecular weight of a full-length antibody, particularly by converting it into an sc(Fv)$_2$.
20   [0021]

In a preferred embodiment, the antibodies of the present invention comprise modified antibodies, such as chimeric antibodies and humanized antibodies that bind to Mpl.   These modified antibodies can be produced by known methods.

Chimeric antibodies are antibodies prepared by combining sequences derived from
25   different animal species, and include for example, antibodies comprising the heavy chain and light chain variable regions of a murine antibody, and the heavy chain and light chain constant regions of a human antibody.   Chimeric antibodies can be prepared by known methods.   For example, a DNA encoding the V region of an antibody is linked to a DNA encoding the C region of a human antibody, and the construct is inserted into an expression vector and introduced into a
30   host to produce chimeric antibodies.

Humanized antibodies are also referred to as "reshaped human antibodies".   Such a humanized antibody is obtained by transferring the complementarity-determining region (CDR) of an antibody derived from a non-human mammal, for example mouse, to the complementarity-determining region of a human antibody, and the general gene recombination
35   procedure for this is also known (see European Patent Application No. 125023 and WO 96/02576).

Specifically, a DNA sequence designed to link a murine antibody CDR to the framework region (FR) of a human antibody can be synthesized by PCR, using primers prepared from several oligonucleotides containing overlapping portions of both CDR and FR terminal regions (see methods described in WO 98/13388).

5        The human antibody framework region to be linked by CDR is selected in order to form a favorable antigen-binding site in the complementarity-determining region.   Amino acids of the framework region in the antibody variable region may be substituted, as necessary, for the complementarity-determining region of the reshaped human antibody to form a suitable antigen-binding site (Sato, K. *et al.*, Cancer Res. (1993) 53: 851-856).

10       The constant region of a human antibody is used as the C region of a chimeric antibody or humanized antibody.   For example, Cγ1, Cγ2, Cγ3, and Cγ4 can be used as the H chain, and Cκ and Cλ can be used as the L chain.   The human antibody C region may be modified to improve the antibody or the stability of the antibody production.

Generally, chimeric antibodies comprise the variable region of an antibody from a
15       non-human mammal and the constant region derived from a human antibody.   On the other hand, humanized antibodies comprise the complementarity-determining region of an antibody from a non-human mammal, and the framework region and C region derived from a human antibody.

In addition, after a chimeric antibody or a humanized antibody is prepared, amino acids
20       in the variable region (for example, FR) and the constant region may be replaced with other amino acids, and such.

The origin of the variable regions in chimeric antibodies or that of the CDRs in humanized antibodies is not particularly limited, and may be derived from any type of animal. For example, sequences of murine antibodies, rat antibodies, rabbit antibodies, camel antibodies
25       may be used.

In general, it is difficult to chimerize or humanize an antibody without losing the agonistic activity of the original antibody.   Nevertheless, the present invention succeeded in preparing humanized antibodies having agonistic activity equivalent to that of the original murine antibody.

30       Chimeric antibodies and humanized antibodies exhibit lower antigenicity in the human body, and thus are expected to be useful when administered to humans for therapeutic purposes.
[0022]
In one embodiment, the preferred antibodies of the present invention include antibodies that bind to soluble Mpl.   The term "soluble Mpl" herein refers to Mpl molecules excluding
35       those expressed on the cell membrane.   A specific example of a soluble Mpl is an Mpl lacking the entire or a portion of the transmembrane domain.   The transmembrane domain of human

Mpl corresponds to amino acids 492 to 513 in SEQ ID NO: 123.

An antibody that binds to soluble recombinant Mpl can be used in detailed epitope analysis and kinetic analysis of receptor-ligand binding, as well as for assessing the blood concentration and dynamic behavior of the antibody in *in vivo* tests.

In one embodiment, the preferred antibodies of the present invention include antibodies having binding activity against both human and monkey Mpl.   Antibodies having agonistic activity to both human and monkey Mpl are expected to be highly useful since the dynamic behavior and *in vivo* effects of the antibody, which are generally difficult to determine in human body, can be examined with monkeys.

The present invention also provides antibodies having agonistic activity to human Mpl and monkey Mpl.   Such antibodies may also have binding activity or agonistic activity against Mpl from animals other than humans and monkeys (for example, mice).

In addition, the antibodies of the present invention include antibodies with TPO agonistic activity (agonistic activity against Mpl) of EC50 = 100 nM or lower, preferably EC50 = 30 nM or lower, more preferably EC50 = 10 nM or lower.

The agonistic activity can be determined by methods known to those skilled in the art, for example, by the method described below.
The sequences for human Mpl (Palacios *et al.*, Cell (1985) 41: 727-734; GenBank Accession NO. NM_005373), cynomolgus monkey Mpl (SEQ ID NO: 157), and mouse Mpl (GenBank Accession NO. NM_010823) are already known.

In addition, the present invention includes antibodies whose binding activities to soluble Mpl are $KD = 10^{-6}$ M or lower, preferably $KD = 10^{-7}$ M or lower, and more preferably $KD = 10^{-8}$ M or lower.

[0023]

In the present invention, whether the binding activity of an antibody to soluble recombinant Mpl is $KD = 10^{-6}$ M or lower can be determined by methods known to those skilled in the art.   For example, the activity can be determined using surface plasmon resonance with Biacore.   Specifically, soluble MPL-Fc protein is immobilized onto sensor chips.   Reaction rate constant can be determined by assessing the interaction between the antibody and the soluble Mpl-Fc protein.   The binding activity can be evaluated by ELISA (enzyme-linked immunosorbent assays), EIA (enzyme immunoassays), RIA (radio immunoassays), or fluorescent antibody techniques.   For example, in enzyme immunoassays, a sample containing a test antibody, such as purified antibody or culture supernatant of cells producing the test antibody, is added to a plate coated with an antigen to which the test antibody can bind.   After incubating the plate with a secondary antibody labeled with an enzyme such as alkaline phosphatase, the plate is washed and an enzyme substrate such as p-nitrophenyl phosphate is added.   The

antigen-binding activity can then be evaluated by determining the absorbance.

There is no specific limitation as to the upper limit of the binding activity; for example, the upper limit may be set within a technically feasible range by those skilled in the art. However, the technically feasible range may expand with the advancement of technology.

5    [0024]

In an embodiment, the preferred antibodies of the present invention include antibodies recognizing epitopes that are recognized by any one of the antibodies indicated in (I) to (IX) below.    The antibody of any one of (I) to (IX) is preferably a minibody.

[0025]

10·  (I)

Antibody comprising a VH that has CDR1, 2, and 3 comprising the amino acid sequences according to SEQ ID NOs in any one of (1) to (19) indicated below (name of each antibody and the H chain CDR contained in the antibody are indicated inside the parentheses):

(1) SEQ ID NOs: 3, 4, and 5 (VA7: H chain CDR1, 2, and 3),

15   (2) SEQ ID NOs: 6, 7, and 8 (VA130: H chain CDR1, 2, and 3),

(3) SEQ ID NOs: 9, 10, and 11 (VA259: H chain CDR1, 2, and 3),

(4) SEQ ID NOs: 12, 13, and 14 (VB17B: H chain CDR1, 2, and 3),

(5) SEQ ID NOs: 15, 16, and 17 (VB12B: H chain CDR1, 2, and 3),

(6) SEQ ID NOs: 18, 19, and 20 (VB140: H chain CDR1, 2, and 3),

20   (7) SEQ ID NOs: 21, 22, and 23 (VB33: H chain CDR1, 2, and 3),

(8) SEQ ID NOs: 24, 25, and 26 (VB45B: H chain CDR1, 2, and 3),

(9) SEQ ID NOs: 27, 28, and 29 (V8B: H chain CDR1, 2, and 3),

(10) SEQ ID NOs: 30, 31, and 32 (VB115: H chain CDR1, 2, and 3),

(11) SEQ ID NOs: 33, 34, and 35 (V14B: H chain CDR1, 2, and 3),

25   (12) SEQ ID NOs: 36, 37, and 38 (V22B: H chain CDR1, 2, and 3),

(13) SEQ ID NOs: 39, 40, and 41 (VB16: H chain CDR1, 2, and 3),

(14) SEQ ID NOs: 42, 43, and 44 (VB157: H chain CDR1, 2, and 3),

(15) SEQ ID NOs: 45, 46, and 47 (VB4B: H chain CDR1, 2, and 3),

(16) SEQ ID NOs: 48, 49, and 50 (VB51: H chain CDR1, 2, and 3),

30   (17) SEQ ID NOs: 51, 52, and 53 (AB317: H chain CDR1, 2, and 3),

(18) SEQ ID NOs: 54, 55, and 56 (AB324: H chain CDR1, 2, and 3),

(19) SEQ ID NOs: 57, 58, and 59 (TA136: H chain CDR1, 2, and 3).

[0026]

(II)

35   Antibody comprising a VL which has CDR1, 2, and 3 comprising the amino acid sequences according to SEQ ID NOs in any one of (1) to (19) indicated below (name of each

antibody and the L chain CDR in the antibody are indicated inside the parentheses):

(1) SEQ ID NOs: 60, 61, and 62 (VA7: L chain CDR1, 2, and 3),

(2) SEQ ID NOs: 63, 64, and 65 (VA130: L chain CDR1, 2, and 3),

(3) SEQ ID NOs: 66, 67, and 68 (VA259: L chain CDR1, 2, and 3),

5   (4) SEQ ID NOs: 69, 70, and 71 (VB17B: L chain CDR1, 2, and 3),

(5) SEQ ID NOs: 72, 73, and 74 (VB12B: L chain CDR1, 2, and 3),

(6) SEQ ID NOs: 75, 76, and 77 (VB140: L chain CDR1, 2, and 3),

(7) SEQ ID NOs: 78, 79, and 80 (VB33: L chain CDR1, 2, and 3),

(8) SEQ ID NOs: 81, 82, and 83 (VB45B: L chain CDR1, 2, and 3),

10   (9) SEQ ID NOs: 84, 85, and 86 (V8B: L chain CDR1, 2, and 3),

(10) SEQ ID NOs: 87, 88, and 89 (VB115: L chain CDR1, 2, and 3),

(11) SEQ ID NOs: 90, 91, and 92 (V14B: L chain CDR1, 2, and 3),

(12) SEQ ID NOs: 93, 94, and 95 (VB22B: L chain CDR1, 2, and 3),

(13) SEQ ID NOs: 96, 97, and 98 (VB16: L chain CDR1, 2, and 3),

15   (14) SEQ ID NOs: 99, 100, and 101 (VB157: L chain CDR1, 2, and 3),

(15) SEQ ID NOs: 102, 103, and 104 (VB4B: L chain CDR1, 2, and 3),

(16) SEQ ID NOs: 105, 106, and 107 (VB51: L chain CDR1, 2, and 3),

(17) SEQ ID NOs: 108, 109, and 110 (AB317: L chain CDR1, 2, and 3),

(18) SEQ ID NOs: 111, 112, and 113 (AB324: L chain CDR1, 2, and 3),

20   (19) SEQ ID NOs: 114, 115, and 116 (TA136: L chain CDR1, 2, and 3).

[0027]

(III)

Antibody comprising a VH that comprises an amino acid sequence of the SEQ ID NO in any one of (1) to (19):

25   (1) SEQ ID NO: 124 (VA7: VH),

(2) SEQ ID NO: 126 (VA130: VH),

(3) SEQ ID NO: 128 (VA259: VH),

(4) SEQ ID NO: 130 (VB17B: VH),

(5) SEQ ID NO: 132 (VB12B: VH),

30   (6) SEQ ID NO: 134 (VB140: VH),

(7) SEQ ID NO: 136 (VB33: VH),

(8) SEQ ID NO: 138 (VB45B: VH),

(9) SEQ ID NO: 140 (V8B: VH),

(10) SEQ ID NO: 142 (VB115: VH),

35   (11) SEQ ID NO: 144 (VB14B: VH),

(12) SEQ ID NO: 118 (VB22B: VH),

(13) SEQ ID NO: 146 (VB16: VH),

(14) SEQ ID NO: 148 (VB157: VH),

(15) SEQ ID NO: 150 (VB4B: VH),

(16) SEQ ID NO: 152 (VB51: VH),

(17) SEQ ID NO: 155 (AB317: VH),

(18) SEQ ID NO: 159 (AB324: VH),

(19) SEQ ID NO: 162 (TA136: VH).

[0028]

(IV)

Antibody comprising a VL that comprises an amino acid sequence of the SEQ ID NO in any one of (1) to (19):

(1) SEQ ID NO: 125 (VA7: VL),

(2) SEQ ID NO: 127 (VA130: VL),

(3) SEQ ID NO: 129 (VA259: VL),

(4) SEQ ID NO: 131 (VB17B: VL),

(5) SEQ ID NO: 133 (VB12B: VL),

(6) SEQ ID NO: 135 (VB140: VL),

(7) SEQ ID NO: 137 (VB33: VL),

(8) SEQ ID NO: 139 (VB45B: VL),

(9) SEQ ID NO: 141 (VB8B: VL),

(10) SEQ ID NO: 143 (VB115: VL),

(11) SEQ ID NO: 145 (VB14B: VL),

(12) SEQ ID NO: 120 (VB22B: VL),

(13) SEQ ID NO: 147 (VB16: VL),

(14) SEQ ID NO: 149 (VB157: VL),

(15) SEQ ID NO: 151 (VB4B: VL),

(16) SEQ ID NO: 153 (VB51: VL),

(17) SEQ ID NO: 157 (AB317: VL),

(18) SEQ ID NO: 161 (AB324: VL),

(19) SEQ ID NO: 163 (TA136: VL).

[0029]

(V)

Antibody comprising a VH and VL according to any one of (1) to (19):

(1) SEQ ID NOs: 3, 4, and 5 (VA7: H chain CDR1, 2, and 3); SEQ ID NOs: 60, 61, and 62 (VA7: L chain CDR1, 2, and 3),

(2) SEQ ID NOs: 6, 7, and 8 (VA130: H chain CDR1, 2, and 3), SEQ ID NOs: 63, 64, and 65

(VA130: L chain CDR1, 2, and 3),

(3) SEQ ID NOs: 9, 10, and 11 (VA259: H chain CDR1, 2, and 3); SEQ ID NOs: 66, 67, and 68 (VA259: L chain CDR1, 2, and 3),

(4) SEQ ID NOs: 12, 13, and 14 (VB17B: H chain CDR1, 2, and 3); SEQ ID NOs: 69, 70, and 71 (VB17B: L chain CDR1, 2, and 3),

(5) SEQ ID NOs: 15, 16, and 17 (VB12B: H chain CDR1, 2, and 3); SEQ ID NOs: 72, 73, and 74 (VB12B: L chain CDR1, 2, and 3),

(6) SEQ ID NOs: 18, 19, and 20 (VB140: H chain CDR1, 2, and 3); SEQ ID NOs: 75, 76, and 77 (VB140: L chain CDR1, 2, and 3),

(7) SEQ ID NOs: 21, 22, and 23 (VB33: H chain CDR1, 2, and 3); SEQ ID NOs: 78, 79, and 80 (VB33: L chain CDR1, 2, and 3),

(8) SEQ ID NOs: 24, 25, and 26 (VB45B: H chain CDR1, 2, and 3); SEQ ID NOs: 81, 82, and 83 (VB45B: L chain CDR1, 2, and 3),

(9) SEQ ID NOs: 27, 28, and 29 (V8B: H chain CDR1, 2, and 3); SEQ ID NOs: 84, 85, and 86 (V8B: L chain CDR1, 2, and 3),

(10) SEQ ID NOs: 30, 31, and 32 (VB115: H chain CDR1, 2, and 3); SEQ ID NOs: 87, 88, and 89 (VB115: L chain CDR1, 2, and 3),

(11) SEQ ID NOs: 33, 34, and 35 (V14B: H chain CDR1, 2, and 3); SEQ ID NOs: 90, 91, and 92 (VB14B: L chain CDR1, 2, and 3),

(12) SEQ ID NOs: 36, 37, and 38 (V22B: H chain CDR1, 2, and 3); SEQ ID NOs: 93, 94, and 95 (VB22B,: L chain CDR1, 2, and 3),

(13) SEQ ID NOs: 39, 40, and 41 (VB16: H chain CDR1, 2, and 3); SEQ ID NOs: 96, 97, and 98 (VB16: L chain CDR1, 2, and 3),

(14) SEQ ID NOs: 42, 43, and 44 (VB157: H chain CDR1, 2, and 3); SEQ ID NOs: 99, 100, and 101 (VB157: L chain CDR1, 2, and 3),

(15) SEQ ID NOs: 45, 46, and 47 (VB4B: H chain CDR1, 2, and 3); SEQ ID NOs: 102, 103, and 104 (VB4B: L chain CDR1, 2, and 3),

(16) SEQ ID NOs: 48, 49, and 50 (VB51: H chain CDR1, 2, and 3); SEQ ID NOs: 105, 106, and 107 (VB51: L chain CDR1, 2, and 3),

(17) SEQ ID NOs: 51, 52, and 53 (AB317: H chain CDR1, 2, and 3); SEQ ID NOs: 108, 109, and 110 (AB317: L chain CDR1, 2, and 3),

(18) SEQ ID NOs: 54, 55, and 56 (AB324: H chain CDR1, 2, and 3); SEQ ID NOs: 111, 112, and 113 (AB324: L chain CDR1, 2, and 3),

(19) SEQ ID NOs: 57, 58, and 59 (TA136: H chain CDR1, 2, and 3); SEQ ID NOs: 114, 115, and 116 (TA136: L chain CDR1, 2, and 3).

[0030]

(VI)

Antibody comprising a VH and a VL that comprise the amino acid sequences according to SEQ ID NOs in any one of (1) to (19) indicated below:

(1) SEQ ID NO: 124 (VA7: VH), SEQ ID NO: 125 (VA7: VL),

(2) SEQ ID NO: 126 (VA130: VH), SEQ ID NO: 127 (VA130: VL),

(3) SEQ ID NO: 128 (VA259: VH), SEQ ID NO: 129 (VA259: VL),

(4) SEQ ID NO: 130 (VB17B: VH), SEQ ID NO: 131 (VB17B: VL),

(5) SEQ ID NO: 132 (VB12B: VH), SEQ ID NO: 133 (VB12B: VL),

(6) SEQ ID NO: 134 (VB140: VH), SEQ ID NO: 135 (VB140: VL),

(7) SEQ ID NO: 136 (VB33: VH), SEQ ID NO: 137 (VB33: VL),

(8) SEQ ID NO: 138 (VB45B: VH), SEQ ID NO: 139 (VB45B: VL),

(9) SEQ ID NO: 140 (V8B: VH), SEQ ID NO: 141 (VB8B: VL),

(10) SEQ ID NO: 142 (VB115: VH), SEQ ID NO: 143 (VB115: VL),

(11) SEQ ID NO: 144 (VB14B: VH), SEQ ID NO: 145 (VB14B: VL),

(12) SEQ ID NO: 118 (VB22B: VH), SEQ ID NO: 120 (VB22B: VL),

(13) SEQ ID NO: 146 (VB16: VH), SEQ ID NO: 147 (VB16: VL),

(14) SEQ ID NO: 148 (VB157: VH), SEQ ID NO: 149 (VB157: VL),

(15) SEQ ID NO: 150 (VB4B: VH), SEQ ID NO: 151 (VB4B: VL),

(16) SEQ ID NO: 152 (VB51: VH), SEQ ID NO: 153 (VB51: VL),

(17) SEQ ID NO: 155 (AB317: VH), SEQ ID NO: 157 (AB317: VL),

(18) SEQ ID NO: 159 (AB324: VH), SEQ ID NO: 161 (AB324: VL),

(19) SEQ ID NO: 162 (TA136: VH), SEQ ID NO: 163 (TA136: VL).

[0031]

(VII)

Antibody comprising the amino acid sequence of SEQ ID NO: 122 (VB22B: scFv).

[0032]

(VIII)

Humanized antibody comprising an amino acid sequence according to any one of SEQ ID NO: 2 (VB22B: sc(Fv)$_2$).

[0033]

(IX)

An antibody comprising an amino acid sequence of any one of (I) to (VIII) indicated above, in which one or more amino acids have been substituted, deleted, added, and/or inserted, wherein the antibody has activity equivalent to that of the antibody of any one of (I) to (V).

[0034]

Herein, the phrase "functionally equivalent" means that an antibody of interest has a

biological or biochemical activity comparable to that of an antibody of the present invention. Such activities include, for example, binding activities and agonistic activities.

[0035]

Methods for preparing polypeptides functionally equivalent to a certain polypeptide are
5   well known to those skilled in the art, and include methods of introducing mutations into polypeptides.   For example, those skilled in the art can prepare an antibody functionally equivalent to the antibodies of the present invention by introducing appropriate mutations into the antibody using site-directed mutagenesis (Hashimoto-Gotoh, T. *et al.* Gene (1995) 152: 271-275; Zoller, MJ, and Smith, M. Methods Enzymol. (1983) 100: 468-500; Kramer, W. *et al.*,
10  Nucleic Acids Res. (1984) 12: 9441-9456; Kramer, W. and Fritz HJ, Methods Enzymol. (1987) 154: 350-367; Kunkel, TA, Proc. Natl. Acad. Sci. USA (1985) 82: 488-492; Kunkel, Methods Enzymol. (1988) 85: 2763-2766), or such.   Amino acid mutations may occur naturally.   Thus, the present invention also comprises antibodies functionally equivalent to the antibodies of the present invention and comprising the amino acid sequences of these antibodies, in which one or
15  more amino acids is mutated.   Generally, the number of amino acids that are mutated is 50 amino acids or less, preferably 30 or less, more preferably 10 or less (for example, five amino acids or less).

[0036]

An amino acid is preferably substituted for a different amino acid(s) that allows the
20  properties of the amino acid side-chain to be conserved.   Examples of amino acid side chain properties are: hydrophobic amino acids (A, I, L, M, F, P, W, Y, and V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, and T), amino acids comprising the following side chains: aliphatic side chains (G, A, V, L, I, and P); hydroxyl-containing side chains (S, T, and Y); sulfur-containing side chains (C and M); carboxylic acid- and amide-containing side chains (D,
25  N, E, and Q); basic side chains (R, K, and H); aromatic ring-containing side chains (H, F, Y, and W) (amino acids are represented by one-letter codes in parentheses).

[0037]

A polypeptide comprising a modified amino acid sequence, in which one or more amino acid residues is deleted, added, and/or replaced with other amino acids, is known to retain its
30  original biological activity (Mark, D. F. *et al.*, Proc. Natl. Acad. Sci. USA (1984) 81: 5662-5666; Zoller, M. J. & Smith, M. Nucleic Acids Research (1982) 10: 6487-6500; Wang, A. *et al.*, Science 224, 1431-1433; Dalbadie-McFarland, G. *et al.*, Proc. Natl. Acad. Sci. USA (1982) 79: 6409-6413).

[0038]

35  Fusion proteins containing antibodies that comprise the amino acid sequence of an antibody of the present invention, in which two or more amino acid residues have been added,

are included in the present invention.   The fusion protein results from a fusion between one of the above antibodies and a second peptide or protein, and is included in the present invention. The fusion protein can be prepared by ligating a polynucleotide encoding an antibody of the present invention and a polynucleotide encoding a second peptide or polypeptide in frame,

5     inserting this into an expression vector, and expressing the fusion construct in a host.   Some techniques known to those skilled in the art are available for this purpose.   The partner peptide or polypeptide to be fused with an antibody of the present invention may be a known peptide, for example, FLAG (Hopp, T. P. *et al.*, BioTechnology (1988) 6: 1204-1210), 6x His consisting of six His (histidine) residues, 10x His, influenza hemagglutinin (HA), human c-myc fragment,

10    VSV-GP fragment, p18HIV fragment, T7-tag, HSV-tag, E-tag, SV40 T antigen fragment, lck tag, α-tubulin fragment, B-tag, Protein C fragment.   Other partner polypeptides to be fused with the antibodies of the present invention include, for example, GST (glutathione-S-transferase), HA (influenza hemagglutinin), immunoglobulin constant region, β-galactosidase, and MBP (maltose-binding protein).   A polynucleotide encoding one of these commercially available

15    peptides or polypeptides can be fused with a polynucleotide encoding an antibody of the present invention.   The fusion polypeptide can be prepared by expressing the fusion construct.
      [0039]
      As described below, the antibodies of the present invention may differ in amino acid sequence, molecular weight, isoelectric point, presence/absence of sugar chains, and

20    conformation depending on the cell or host producing the antibody, or purification method. However, a resulting antibody is included in the present invention, as long as it is functionally equivalent to an antibody of the present invention.   For example, when an antibody of the present invention is expressed in prokaryotic cells, for example *E. coli*, a methionine residue is added to the N terminus of the original antibody amino acid sequence.   Such antibodies are

25    included in the present invention.
      [0040]
      An antibody that recognizes an epitope recognized by the antibody according to any one of (I) to (IX) indicated above is expected to have a high agonistic activity.   Such antibodies can be prepared by methods known to those skilled in the art.   The antibody can be prepared by, for

30    example, determining the epitope recognized by the antibody according to any one of (I) to (IX) by conventional methods, and using a polypeptide comprising one of the epitope amino acid sequences as an immunogen.   Alternatively, the antibody can be prepared by determining the epitopes of conventionally prepared antibodies and selecting an antibody that recognizes the epitope recognized by an antibody of any one of (I) to (IX).

35            [0041]
      In the present invention, a particularly preferred antibody is an antibody that recognizes

the epitope recognized by the antibody comprising the amino acid sequence of SEQ ID NO: 2. The antibody comprising the amino acid sequence of SEQ ID NO: 2 is predicted to recognize the region from Glu 26 to Leu 274, preferably the region from Ala 189 to Gly 245, more preferably the region from Gln 213 to Ala 231 of human Mpl.   Thus, antibodies recognizing the region of

5      amino acids 26 to 274, or amino acids 189 to 245, or amino acids 213 to 231 of human Mpl are also included in the present invention.

[0042]

Antibodies recognizing regions of amino acids 26 to 274, amino acids 189 to 245, or amino acids 213 to 231 of the human Mpl amino acid sequence (SEQ ID NO: 123) can be

10     obtained by methods known to those skilled in the art.   Such antibodies can be prepared by, for example, using a peptide comprising amino acids 26 to 274, amino acids 189 to 245, or amino acids 213 to 231 of the human Mpl amino acid sequence (SEQ ID NO: 123) as an immunogen. Alternatively, such antibodies can be prepared by determining the epitope of a conventionally prepared antibody and selecting an antibody that recognizes the same epitope recognized by an

15     antibody of the present invention.

[0043]

The present invention provides antibodies described above in (I) to (IX).   In an embodiment of the present invention, a preferred antibody is the one shown in (V), a more preferred antibody is the one shown in (VI), and a still more preferred is the one shown in (VII)

20     or (VIII).

[0044]

The present invention also provides vectors comprising polynucleotides encoding the antibodies of the present invention, or polynucleotides which hybridize under stringent conditions to the polynucleotides of the present invention and encode antibodies having activities

25     equivalent to those of the antibodies of the present invention.   The polynucleotides of the present invention are polymers comprising multiple bases or base pairs of deoxyribonucleic acids (DNA) or ribonucleic acids (RNA), and are not particularly limited, as long as they encode the antibodies of the present invention.   They may also contain non-natural nucleotides.   The polynucleotides of the present invention can be used to express antibodies using genetic

30     engineering techniques.   The polynucleotides of this invention can also be used as probes in the screening of antibodies functionally equivalent to the antibodies of the present invention. Specifically, DNAs that hybridize under stringent conditions to a polynucleotide encoding an antibody of the present invention, and encode antibodies having activity equivalent to those of the antibodies of the present invention can be obtained by techniques such as hybridization and

35     gene amplification (for example, PCR), using a polynucleotide of the present invention or a portion thereof as a probe.   Such DNAs are also included in the polynucleotides of the present

invention.    Hybridization techniques are well known to those skilled in the art (Sambrook, J *et al.*, Molecular Cloning 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. press, 1989).    Such hybridization conditions include, for example, conditions of low stringency.    Examples of conditions of low stringency include post-hybridization washing in 0.1x SSC and 0.1% SDS at

5    42°C, and preferably in 0.1x SSC and 0.1% SDS at 50°C.    More preferable hybridization conditions include those of high stringency.    Highly stringent conditions include, for example, washing in 5x SSC and 0.1% SDS at 65°C.    In these conditions, the higher the temperature, the higher the expectation of efficiently obtaining polynucleotides with a high homology.    However, several factors, such as temperature and salt concentration, can influence hybridization

10    stringency, and those skilled in the art can suitably select these factors to accomplish similar stringencies.

[0045]

Antibodies that are encoded by polynucleotides obtained by the hybridization and gene amplification techniques, and are functionally equivalent to the antibodies of the present

15    invention generally exhibit high homology to the antibodies of the this invention at the amino acid level.    The antibodies of the present invention include antibodies that are functionally equivalent to the antibodies of the present invention, and exhibit high amino acid sequence homology to the antibodies of this invention.    The term "high homology" generally means identity at the amino acid level of at least 50% or higher, preferably 75% or higher, more

20    preferably 85% or higher, still more preferably 95% or higher.    Polypeptide homology can be determined by the algorithm described in the report: Wilbur, W. J. and Lipman, D. J. Proc. Natl. Acad. Sci. USA (1983) 80: 726-730.

[0046]

When *E. coli* is used as a host, there is no particular limitation as to the type of vector of

25    the present invention, as long as the vector contains an "ori" responsible for its replication in *E. coli* and a marker gene.    The "ori" ensures the amplification and mass production of the vector in *E. coli* (for example, JM109, DH5α, HB101, and XL1Blue).    The marker gene is used to select the *E. coli* transformants (for example, a drug resistance gene selected by an appropriate drug such as ampicillin, tetracycline, kanamycin, and chloramphenicol).    The vectors include,

30    for example, M13 vectors, pUC vectors, pBR322, pBluescript, and pCR-Script.    In addition to the above vectors, for example, pGEM-T, pDIRECT, and pT7 can also be used for the subcloning and excision of cDNAs.

[0047]

An expression vector is especially useful for the type of vectors of the present invention.

35    When an expression vector is expressed, for example, in *E. coli*, it should have the above characteristics in order to be amplified in *E. coli*.    Additionally, when *E. coli*, such as JM109,

DH5α, HB101, or XL1-Blue are used as the host cell, the vector preferably has a promoter, for example, lacZ promoter (Ward *et al.* Nature (1989) 341: 544-546; FASEB J. (1992) 6: 2422-2427), araB promoter (Better *et al.,* Science (1988) 240: 1041-1043), or T7 promoter, that allows efficient expression of the desired gene in *E. coli.* Other examples of the vectors include pGEX-5X-1 (Pharmacia), "QIAexpress system" (QIAGEN), pEGFP, and pET (where BL21, a strain expressing T7 RNA polymerase, is preferably used as the host).

[0048]

Furthermore, the vector may comprise a signal sequence for polypeptide secretion. When producing polypeptides into the periplasm of *E. coli*, the pelB signal sequence (Lei, S. P. *et al.* J. Bacteriol. (1987) 169: 4379) may be used as a signal sequence for polypeptide secretion. For example, calcium chloride methods or electroporation methods may be used to introduce the vector into a host cell.

[0049]

In addition to *E. coli*, expression vectors derived from mammals (e.g., pCDNA3 (Invitrogen), pEGF-BOS (Nucleic Acids Res. (1990) 18 (17): 5322), pEF, pCDM8), insect cells (e.g., "Bac-to-BAC baculovirus expression system" (GIBCO-BRL), pBacPAK8), plants (e.g., pMH1, pMH2), animal viruses (e.g., pHSV, pMV, pAdexLcw), retroviruses (e.g., pZIPneo), yeasts (e.g., "Pichia Expression Kit" (Invitrogen), pNV11, SP-Q01), and *Bacillus subtilis* (e.g., pPL608, pKTH50) may also be used as a vector of the present invention.

[0050]

In order to express proteins in animal cells such as CHO, COS, and NIH3T3 cells, the vector preferably has a promoter necessary for expression in such cells, for example, an SV40 promoter (Mulligan *et al.* Nature (1979) 277: 108), MMLV-LTR promoter, EF1α promoter (Mizushima *et al.* Nucleic Acids Res. (1990) 18: 5322), CMV promoter, etc.). It is even more preferable that the vector also carries a marker gene for selecting transformants (for example, a drug-resistance gene selected by a drug such as neomycin and G418). Examples of vectors with such characteristics include pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV, and pOP13, and such.

[0051]

In addition, to stably express a gene and amplify the gene copy number in cells, CHO cells that are defective in the nucleic acid synthesis pathway are introduced with a vector containing a DHFR gene (for example, pCHOI) to compensate for the defect, and the copy number is amplified using methotrexate (MTX). Alternatively, a COS cell, which carries an SV40 T antigen-expressing gene on its chromosome, can be transformed with a vector containing the SV40 replication origin (for example, pcD) for transient gene expression. The replication origin may be derived from polyoma virus, adenovirus, bovine papilloma virus

(BPV), and such.    Furthermore, to increase the gene copy number in host cells, the expression vector may contain, as a selection marker, aminoglycoside transferase (APH) gene, thymidine kinase (TK) gene, *E. coli* xanthine guanine phosphoribosyl transferase (Ecogpt) gene, dihydrofolate reductase (dhfr) gene, and such.

[0052]

Next, the vector is introduced into a host cell.    The host cells into which the vector is introduced are not particularly limited, for example, *E. coli* and various animal cells are available for this purpose.    The host cells may be used, for example, as a production system to produce and express the antibodies of the present invention.    *In vitro* and *in vivo* production systems are available for polypeptide production systems.    Production systems that use eukaryotic cells or prokaryotic cells are examples of *in vitro* production systems.

[0053]

Eukaryotic cells that can be used are, for example, animal cells, plant cells, and fungal cells.    Known animal cells include: mammalian cells, for example, CHO (J. Exp. Med. (1995) 108: 945), COS, 3T3, myeloma, BHK (baby hamster kidney), HeLa, Vero, amphibian cells such as *Xenopus laevis* oocytes (Valle, *et al.,* Nature (1981) 291: 358-340), or insect cells (e.g., Sf9, Sf21, and Tn5).    In the present invention, CHO-DG44, CHO-DXB11, COS7 cells, and BHK cells can be suitably used.    Among animal cells, CHO cells are particularly favorable for large-scale expression.    Vectors can be introduced into a host cell by, for example, calcium phosphate methods, the DEAE-dextran methods, methods using cationic liposome DOTAP (Boehringer-Mannheim), electroporation methods, lipofection methods.

[0054]

Plant cells include, for example, *Nicotiana tabacum*-derived cells known as a protein production system.    Calluses may be cultured from these cells.    Known fungal cells include yeast cells, for example, genus *Saccharomyces* such as *Saccharomyces cerevisiae* and *Saccharomyces pombe*; and filamentous fungi, for example, genus *Aspergillus* such as *Aspergillus niger*.

[0055]

Bacterial cells can be used in the prokaryotic production systems.    Examples of bacterial cells include *E. coli* (for example, JM109, DH5α, HB101 and such); and *Bacillus subtilis*.

[0056]

Next, the above host cells are cultured.    Antibodies can be obtained by transforming the cells with a polynucleotide of interest and *in vitro* culturing of these transformants. Transformants can be cultured using known methods.    For example, DMEM, MEM, RPMI 1640, or IMDM may be used as the culture medium for animal cells, and may be used with or

without serum supplements such as FBS or fetal calf serum (FCS).    Serum-free cultures are also acceptable.    The preferred pH is about 6 to 8 during the course of culturing.    Incubation is carried out typically at a temperature of about 30 to 40°C for about 15 to 200 hours. Medium is exchanged, aerated, or agitated, as necessary.

5          [0057]

On the other hand, production systems using animal or plant hosts may be used as systems for producing polypeptides *in vivo*.    For example, a polynucleotide of interest is introduced into an animal or plant and the polypeptide is produced in the body of the animal or plant and then recovered.    The "hosts" of the present invention includes such animals and

10    plants.

[0058]

Animals to be used for the production system include mammals or insects.    Mammals such as goats, pigs, sheep, mice, and cattle may be used (Vicki Glaser SPECTRUM Biotechnology Applications (1993)).    Alternatively, the mammals may be transgenic animals.

15          For example, a polynucleotide of interest is prepared as a fusion gene with a gene encoding a polypeptide specifically produced in milk, such as the goat β-casein gene.    DNA fragments containing the fusion gene are injected into goat embryos, which are then introduced back to female goats.    The desired antibody can be obtained from milk produced by the transgenic goats, which are born from the goats that received the embryos, or from their

20    offspring.    Appropriate hormones may be administered to increase the volume of milk containing the antibody produced by the transgenic goats (Ebert, K.M. *et al.*, Bio/Technology (1994) 12: 699-702).

[0059]

Insects, such as silkworms, may also be used.    Baculoviruses carrying a polynucleotide

25    encoding an antibody of interest can be used to infect silkworms, and the antibody of interest can be obtained from the body fluids (Susumu, M. *et al.*, Nature (1985) 315: 592-594).

[0060]

Plants used in the production system include, for example, tobacco.    When tobacco is used, a polynucleotide encoding an antibody of interest is inserted into a plant expression vector,

30    for example, pMON 530, and then the vector is introduced into a bacterium, such as *Agrobacterium tumefaciens*.    The bacteria are then used to infect tobacco such as Nicotiana tabacum, and the desired antibodies can be recovered from the leaves (Julian K.-C. Ma *et al.*, Eur. J. Immunol. (1994) 24: 131-138).

[0061]

35          The resulting antibody may be isolated from the inside or outside (such as the medium) of host cells, and purified as a substantially pure and homogenous antibody.    Methods are not

limited to any specific method and any standard method for isolating and purifying antibodies may be used.   Polypeptides may be isolated and purified, by selecting an appropriate combination of, for example, chromatographic columns, filtration, ultrafiltration, salting out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide

5    gel electrophoresis, isoelectric focusing, dialysis, recrystallization, and others.

Chromatographies include, for example, affinity chromatographies, ion exchange chromatographies, hydrophobic chromatographies, gel filtrations, reverse-phase chromatographies, and adsorption chromatographies (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak *et al.*, Cold Spring Harbor

10   Laboratory Press, 1996).   These chromatographies can be carried out using liquid phase chromatographies such as HPLC and FPLC.   Examples of the affinity chromatography columns include protein A columns and protein G columns.   Examples of the proteins A columns include Hyper D, POROS, and Sepharose F. F. (Pharmacia).

[0062]

15   An antibody can be modified freely and peptide portions deleted by treating the antibody with an appropriate protein modifying enzyme before or after antibody purification. Such protein modifying enzymes include, for example, trypsins, chymotrypsins, lysyl endopeptidases, protein kinases, and glucosidases.

[0063]

20   Antibodies that bind to Mpl can be prepared by methods known to those skilled in the art.

For example, monoclonal antibody-producing hybridomas can be essentially generated by known technologies as follows: immunizing animals with Mpl proteins or Mpl-expressing cells as sensitized antigens using conventional immunological methods; fusing the obtained

25   immunocytes with known parental cells by conventional cell fusion methods; and screening for monoclonal antibody-producing cells by conventional methods.

Specifically, monoclonal antibodies can be prepared by the method below.

First, Mpl protein, which is used as a sensitized antigen for preparing antibodies, is prepared by expressing the Mpl gene/amino acid sequence (GenBank accession number:

30   NM_005373).   More specifically, the gene sequence encoding Mpl is inserted into a known expression vector, which is then transfected into an appropriate host cell.   The subject human Mpl protein is purified from the host cell or culture supernatant using known methods.

The purified Mpl protein is then used as a sensitized antigen.   Alternatively, a partial Mpl peptide may be used as a sensitized antigen.   In this case, the partial peptide can also be

35   chemically synthesized based on the amino acid sequence of human Mpl.

[0064]

The epitopes of Mpl molecule that are recognized by an anti-Mpl antibody of the present invention are not limited to a particular epitope, and may be any epitope on the Mpl molecule. Thus, any fragment can be used as an antigen for preparing anti-Mpl antibodies of the present invention, as long as the fragment comprises an epitope of the Mpl molecule.

[0065]

There is no limitation as to the type of mammalian species to be immunized with the sensitized antigen. However, a mammal is preferably selected based on its compatibility with the parental cell to be used in cell fusion. Generally, rodents (for example, mice, rats, and hamsters), rabbits, and monkeys can be used.

[0066]

Animals can be immunized with a sensitized antigen by known methods such as a routine method of injecting a sensitized antigen into a mammal intraperitoneally or subcutaneously. Specifically, the sensitized antigen is diluted appropriately with phosphate-buffered saline (PBS), physiological saline and such, and then suspended. An adequate amount of a conventional adjuvant, for example, Freund's complete adjuvant, is mixed with the suspension, as necessary. An emulsion is then prepared for administering to a mammal several times over a 4- to 21-day interval. An appropriate carrier may be used for the sensitized antigen in immunization.

A mammal is immunized as described above. After a titer increase of target antibody in the serum is confirmed, immunocytes are collected from the mammal and then subjected to cell fusion. Spleen cells are the preferred immunocytes.

[0067]

Mammalian myeloma cells are used as the parental cells to be fused with the above immunocytes. Preferable myeloma cells to be used include various known cell lines, for example, P3 (P3x63Ag8.653) (Kearney JF, et al., J. Immnol. (1979) 123: 1548-1550), P3x63Ag8U.1 (Yelton DE, et al., Current Topics in Microbiology and Immunology (1978) 81: 1-7), NS-1 (Kohler, G. and Milstein, C. Eur. J. Immunol. (1976) 6: 511-519), MPC-11 (Margulies, D. H. et al., Cell (1976) 8: 405-415), SP2/0 (Shulman, M. et al., Nature (1978) 276: 269-270), FO (deSt. Groth, S. F. et al., J. Immunol. Methods (1980) 35: 1-21), S194 (Trowbridge, I. S., J. Exp. Med. (1978) 148: 313-323), and R210 (Galfre, G. et al., Nature (1979) 277: 131-133).

[0068]

Cell fusions between the immunocytes and the myeloma cells as described above can be essentially carried out using known methods, for example, a method by Kohler and Milstein (Kohler, G. and Milstein, C., Methods Enzymol. (1981) 73: 3-46).

[0069]

More specifically, the above-described cell fusions are carried out, for example, in a

conventional culture medium in the presence of a cell fusion-promoting agent.   The
fusion-promoting agents include, for example, polyethylene glycol (PEG) and Sendai virus
(HVJ).   If required, an auxiliary substance such as dimethyl sulfoxide may also be added to
improve fusion efficiency.

5      [0070]

The ratio of immunocytes to myeloma cells may be determined at one's own discretion,
preferably, for example, one myeloma cell for every one to ten immunocytes.   Culture media to
be used for the above cell fusions include, for example, media that are suitable for the growth of
the above myeloma cell lines, such as RPMI 1640 media and MEM media, and other
10     conventional culture media used for this type of cell culture.   In addition, serum supplements
such as fetal calf serum (FCS) may also be used in combination.

[0071]

Cell fusion is carried out as follows.   As described above, predetermined amounts of
immunocytes and myeloma cells are mixed well in the culture medium.   PEG solution (for
15     example, mean molecular weight of about 1,000-6,000) pre-heated to 37°C is added to the cell
suspension typically at a concentration of 30% to 60% (w/v), and mixed to produce fused cells
(hybridomas).   Then, an appropriate culture medium is successively added to the mixture, and
the sample is centrifuged to remove supernatant.   This treatment is repeated several times to
remove the unwanted cell fusion-promoting agent and others that are unfavorable to hybridoma
20     growth.

[0072]

Screening of the resulting hybridomas can be carried out by culturing them in a
conventional selective medium, for example, hypoxanthine, aminopterin, and thymidine (HAT)
medium.   Culturing in the above-descried HAT medium is continued for a period long enough
25     (typically, for several days to several weeks) to kill cells (non-fused cells) other than the desired
hybridomas.   Then, hybridomas are screened for single-cell clones capable of producing the
target antibody by conventional limiting dilution methods.

[0073]

In addition to the method for preparing the above-descried hybridomas by immunizing
30     non-human animals with antigens, preferred human antibodies having binding activity to Mpl
can also be obtained by: sensitizing human lymphocytes with Mpl *in vitro*; and fusing the
sensitized lymphocytes with human myeloma cells capable of dividing permanently (see,
Japanese Patent Application Kokoku Publication No. (JP-B) H01-59878 (examined, approved
Japanese patent application published for opposition)).   Alternatively, it is possible to obtain
35     human antibodies against Mpl from immortalized cells producing anti-Mpl antibodies.   In this
method, the cells producing anti-Mpl antibodies are prepared by administering Mpl as an antigen

to transgenic animals comprising a repertoire of the entire human antibody genes (see, WO 94/25585, WO 93/12227, WO 92/03918, and WO 94/02602).

The monoclonal antibody-producing hybridomas thus prepared can be passaged in a conventional culture medium, and stored in liquid nitrogen over long periods of time.

[0074]

Monoclonal antibodies can be prepared from the above-described hybridomas by, for example, a routine procedure of culturing the hybridomas and obtaining antibodies from the culture supernatants.   Alternatively, monoclonal antibodies can be prepared by injecting the hybridomas into a compatible mammal; growing these hybridomas in the mammal; and obtaining antibodies from the mammal's ascites.   The former method is suitable for preparing highly purified antibodies, while the latter is suitable for preparing antibodies on a large scale.

[0075]

Recombinant antibodies can also be prepared by: cloning an antibody gene from a hybridoma; inserting the gene into an appropriate vector; introducing the vector into a host; and producing the antibodies by using genetic recombination techniques (see, for example, Vandamme, A. M. et al., Eur. J. Biochem. (1990) 192: 767-775).

[0076]

Specifically, an mRNA encoding the variable (V) region of anti-Mpl antibody is isolated from hybridomas producing the anti-Mpl antibodies.   For mRNA isolation, total RNAs are first prepared by conventional methods such as guanidine ultracentrifugation methods (Chirgwin, J. M. et al., Biochemistry (1979) 18: 5294-5299), or acid guanidinium thiocyanate-phenol-chloroform (AGPC) methods (Chomczynski, P. et al., Anal. Biochem. (1987) 162: 156-159), and then the target mRNA is prepared using an mRNA Purification Kit (Pharmacia) and such.   Alternatively, the mRNA can be directly prepared using the QuickPrep mRNA Purification Kit (Pharmacia).

[0077]

A cDNA of the antibody V region is synthesized from the resulting mRNA using reverse transcriptase.   cDNA synthesis is carried out using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Co.), or such.   Alternatively, cDNA can be synthesized and amplified by the 5'-RACE method (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA (1988) 85: 8998-9002; Belyavsky, A. et al., Nucleic Acids Res. (1989) 17: 2919-2932) using the 5'-Ampli FINDER RACE Kit (Clontech) and PCR.

[0078]

Target DNA fragments are purified from the obtained PCR products and then ligated with vector DNAs to prepare recombinant vectors.   The vectors are introduced into E. coli and such, and colonies are selected for preparing the recombinant vector of interest.   The target

DNA nucleotide sequence is then confirmed by conventional methods such as the dideoxynucleotide chain termination method.

Once a DNA encoding the V region of target anti-Mpl antibody is obtained, the DNA is inserted into an expression vector which comprises a DNA encoding the constant region (C region) of a desired antibody.

5

The method for producing anti-Mpl antibodies to be used in the present invention typically comprises the steps of: inserting an antibody gene into an expression vector, so that the gene is expressed under the regulation of expression regulatory regions, such as enhancer and promotor; and transforming host cells with the resulting vectors to express antibodies.

10

For expressing the antibody gene, polynucleotides encoding H chain and L chain, respectively, are inserted into separate expression vectors and co-transfected into a host cell. Alternatively, polynucleotides encoding both H chain and L chain are inserted into a single expression vector and transfected into a host cell (see WO 94/11523).

[0079]

15

The term "agonistic activity" refers to an activity to induce changes in some biological activities through signal transduction into cells and such, due to the binding of an antibody to a receptor antigen.  The biological activities include, for example, proliferation-promoting activities, proliferation activities, viability activities, differentiation-inducing activities, differentiation activities, transcriptional activities, membrane transport activities, binding

20

activities, proteolytic activities, phosphorylation/dephosphorylation activities, oxidation/reduction activities, transfer activities, nucleolytic activities, dehydration activities, cell death-inducing activities, and apoptosis-inducing activities, but is not limited thereto.

The term "agonistic activity against Mpl" typically refers to the activity of promoting the differentiation of megakaryocytes or their parental hemopoietic stem cells into platelets, or

25

the activity of stimulating platelet proliferation.

Agonistic activity can be assayed by methods known to those skilled in the art.   The agonistic activity may be determined using the original activity or a different activity as an indicator.

For example, agonistic activity can be determined by a method using cell growth as an

30

indicator as described in Examples.   More specifically, an antibody whose agonistic activity is to be determined is added to cells which proliferate in an agonist-dependent manner, followed by incubation of the cells.   Then, a reagent such as WST-8, which shows a coloring reaction at specific wavelengths depending on the viable cell count, is added to the culture and absorbance is measured.   The agonistic activity can be determined using the measured absorbance as an

35

indicator.

[0080]

Cells that proliferate in an agonist-dependent manner can also be prepared by methods known to those skilled in the art.   For example, when the antigen is a receptor capable of transducing cell growth signals, cells expressing the receptor may be used.   Alternatively, when the antigen is a receptor that cannot transduce signals, a chimeric receptor consisting of the

5    intracellular domain of a receptor that transduces cell growth signals and the extracellular domain of a receptor that does not transduce cell growth signals can be prepared for cellular expression.   Receptors that transduce cell growth signals include, for example, G-CSF receptors, mpl, neu, GM-CSF receptors, EPO receptors, c-kit, and FLT-3.   Cells that can be used to express a receptor include, for example, BaF3, NFS60, FDCP-1, FDCP-2, CTLL-2, DA-1, and

10   KT-3.

[0081]

There is no limitation as to the type of detection indicators to be used for determining agonistic activity, as long as the indicator can monitor quantitative and/or qualitative changes. For example, it is possible to use cell-free assay indicators, cell-based assay indicators,

15   tissue-based assay indicators, and *in vivo* assay indicators.   Indicators that can be used in cell-free assays include enzymatic reactions, quantitative and/or qualitative changes in proteins, DNAs, or RNAs.   Such enzymatic reactions include, for example, amino acid transfers, sugar transfers, dehydrations, dehydrogenations, and substrate cleavages.   Alternatively, protein phosphorylations, dephosphorylations, dimerizations, multimerizations, hydrolyses, dissociations

20   and such; DNA or RNA amplifications, cleavages, and extensions can be used as the indicator in cell-free assays.   For example, protein phosphorylations downstream of a signal transduction pathway may be used as a detection indicator.   Alterations in cell phenotype, for example, quantitative and/or qualitative alterations in products, alterations in growth activity, alterations in cell number, morphological alterations, or alterations in cellular properties, can be used as the

25   indicator in cell-based assays.   The products include, for example, secretory proteins, surface antigens, intracellular proteins, and mRNAs.   The morphological alterations include, for example, alterations in dendrite formation and/or dendrite number, alteration in cell flatness, alteration in cell elongation/axial ratio, alterations in cell size, alterations in intracellular structure, heterogeneity/homogeneity of cell populations, and alterations in cell density.   Such

30   morphological alterations can be observed under a microscope.   Cellular properties to be used as the indicator include anchor dependency, cytokine-dependent response, hormone dependency, drug resistance, cell motility, cell migration activity, pulsatory activity, and alteration in intracellular substances.   Cell motility includes cell infiltration activity and cell migration activity.   The alterations in intracellular substances include, for example, alterations in enzyme

35   activity, mRNA levels, levels of intracellular signaling molecules such as $Ca^{2+}$ and cAMP, and intracellular protein levels.   When a cell membrane receptor is used, alterations in the cell

proliferating activity induced by receptor stimulation can be used as the indicator.   The
indicators to be used in tissue-based assays include functional alterations adequate for the subject
tissue.   In *in vivo* assays, alterations in tissue weight, alterations in the blood system (for
example, alterations in blood cell counts, protein contents, or enzyme activities), alterations in
electrolyte levels, and alterations in the circulating system (for example, alterations in blood
pressure or heart rate).

[0082]

The methods for measuring such detection indices are not particularly limited.   For
example, absorbance, luminescence, color development, fluorescence, radioactivity, fluorescence
polarization, surface plasmon resonance signal, time-resolved fluorescence, mass, absorption
spectrum, light scattering, and fluorescence resonance energy transfer may be used.   These
measurement methods are known to those skilled in the art and may be selected appropriately
depending on the purpose.   For example, absorption spectra can be obtained by using a
conventional photometer, plate reader, or such; luminescence can be measured with a
luminometer or such; and fluorescence can be measured with a fluorometer or such.   Mass can
be determined with a mass spectrometer.   Radioactivity can be determined with a device such
as a gamma counter depending on the type of radiation.   Fluorescence polarization can be
measured with BEACON (TaKaRa).   Surface plasmon resonance signals can be obtained with
BIACORE.   Time-resolved fluorescence, fluorescence resonance energy transfer, or such can
be measured with ARVO or such.   Furthermore, a flow cytometer can also be used for
measuring.   It is possible to use one of the above methods to measure two or more different
types of detection indices.   A greater number of detection indices may also be examined by
using two or more measurement methods simultaneously and/or consecutively.   For example,
fluorescence and fluorescence resonance energy transfer can be measured at the same time with a
fluorometer.

[0083]

The present invention provides pharmaceutical compositions comprising antibodies of
this invention.   The pharmaceutical compositions comprising antibodies of the present
invention are useful for treating and/or preventing thrombocytopenia and such.   Time required
for the platelet count to recover to the normal level can be shortened by administering an
antibody of the present invention after donation of platelet components.   The amount of platelet
components at the time of blood collection can be increased by pre-administering an antibody of
the present invention.

[0084]

When used as pharmaceutical compositions, the antibodies of the present invention can
be formulated by methods known to those skilled in the art.   For example, the antibodies can be

administered parenterally by injection of a sterile solution or suspension in water or other pharmaceutically acceptable solvents.   For example, the antibodies can be formulated by appropriately combining with pharmaceutically-acceptable carriers or solvents, specifically, sterile water or physiological saline, vegetable oils, emulsifiers, suspending agents, surfactants,

5    stabilizers, flavoring agents, excipients, vehicles, preservatives, binding agents, and such, and mixing at a unit dosage and form required by accepted pharmaceutical implementations.   In such formulations, the amount of the thus obtained active ingredient should be within the required range.

[0085]

10    A sterile composition to be injected can be formulated using a vehicle such as distilled water used for injection, according to standard protocols.

[0086]

Aqueous solutions used for injections include, for example, physiological saline and isotonic solutions comprising glucose or other adjunctive agents such as D-sorbitol, D-mannose,

15    D-mannitol, and sodium chloride.   They may also be combined with an appropriate solubilizing agent such as alcohol, specifically, ethanol, polyalcohol such as propylene glycol or polyethylene glycol, or non-ionic detergent such as polysorbate 80™ or HCO-50, as necessary.

[0087]

Oil solutions include sesame oils and soybean oils, and can be combined with

20    solubilizing agents such as benzyl benzoate or benzyl alcohol.   Injection solutions may also be formulated with buffers, for example, phosphate buffers or sodium acetate buffers; analgesics, for example, procaine hydrochloride; stabilizers, for example, benzyl alcohol or phenol; or anti-oxidants.   The prepared injections are typically aliquoted into appropriate ampules.

[0088]

25    The administration is preferably carried out parenterally, specifically, by injection, intranasal administration, intrapulmonary administration, percutaneous administration, or such. Injections include, for example, intravenous injections, intramuscular injections, intraperitoneal injections, and subcutaneous injections.   The injection solutions can be also administered systemically or locally.

30    [0089]

The administration methods can be selected properly according to the patient's age, condition, and such.   The applied dose of a pharmaceutical composition comprising an antibody or polynucleotide encoding the antibody may be, for example, in the range of 0.0001 to 1,000 mg/kg body weight.   Alternatively, the dosage may be, for example, in the range of 0.001 to

35    100,000 mg/kg body weight.   However, the dosage is not restricted to the values described above.   The dosage and administration methods depend on the patient's weight, age, and

condition, and are appropriately selected by those skilled in the art.

[Examples]

[0090]

The present invention is specifically illustrated below with reference to Examples, but it

5    is not to be construed as being limited thereto.

[Example 1]    Preparation of anti-human Mpl antibodies

1.1 Establishment of Mpl-expressing BaF3 cell lines

BaF3 cell lines expressing the full-length Mpl gene were established to obtain cell lines

that proliferate in a TPO-dependent manner.

10    A full-length human Mpl cDNA (Palacios, R. *et al.*, Cell (1985) 41: 727-734) (GenBank

accession NO. NM_005373) was amplified by PCR.    The cDNA was cloned into a pCOS2

expression vector to construct pCOS2-hMplfull.    The expression vector pCOS2 was

constructed by removing the DHFR gene expression region from pCHOI (Hirata, Y. *et al.*, FEBS

Letter (1994) 356: 244-248), where the expression region of the neomycin resistance gene

15    HEF-VH-gγ1 (Sato, K. *et al.*, Mol Immunol. (1994) 31: 371-381) is inserted.

The cynomolgus monkey Mpl cDNA (SEQ ID NO: 164) was cloned from total RNA

extracted from the bone marrow cells of cynomolgus monkey, using a SMART RACE cDNA

Amplification Kit (Clontech).    The resulting cynomolgus monkey cDNA was inserted into

pCOS2 to construct pCOS2-monkeyMplfull.

20    Then, the full-length mouse Mpl cDNA (GenBank accession NO. NM_010823) was

amplified by PCR, and inserted into pCOS2 to construct pCOS2-mouseMplfull.

Each vector (20 μg) prepared as described above was mixed with BaF3 cells (1 x 10$^7$

cells/mL) suspended in PBS in Gene Pulser cuvettes.    This mixture was then pulsed at 0.33 kV

and 950 μFD using a Gene Pulser II (Bio-Rad).    The BaF3 cells introduced with the above

25    DNAs by electroporation were added to RPMI 1640 medium (Invitrogen) containing 1 ng/mL

mouse interleukin 3 (hereinafter abbreviated as mIL-3; Peprotech), 500 μg/mL Geneticin

(Invitrogen), and 10% FBS (Invitrogen), and selected to establish a human Mpl-expressing BaF3

cell line (hereinafter abbreviated as "BaF3-human Mpl"), monkey Mpl-expressing BaF3 cell line

(hereinafter abbreviated as BaF3-monkey Mpl), and mouse Mpl-expressing BaF3 cell line

30    (hereinafter abbreviated as "BaF3-mouse Mpl").    Following selection, these cells were cultured

and maintained in RPMI 1640 containing 1 ng/mL rhTPO (R&D) and 10% FBS.

[0091]

1.2 Establishment of Mpl-expressing CHO cell lines

CHO cell lines expressing the full-length Mpl gene were established to obtain cell lines

35    to be used for assessing binding activity by flow cytometry.

First, the DHFR gene expression site from pCHOI was inserted into pCXN2 (Niwa, H.

*et al.*, Gene (1991) 108: 193-199) at the HindIII site to prepare a pCXND3 expression vector. The respective Mpl genes were amplified by PCR using pCOS2-hMplfull, pCOS2-monkeyMplfull, and pCOS2-mouseMplfull as templates, and primers with a His-tag sequence. The PCR products were cloned into pCXND3 to construct pCXND3-hMpl-His,

5   pCXND3-monkey Mpl-His, and pCXND3-mouse Mpl-His, respectively.

Vectors thus prepared (25 µg each) were mixed with a PBS suspension of CHO-DG44 cells (1 x 10$^7$ cells/mL) in Gene Pulser cuvettes. The mixture was then pulsed at 1.5 kV and 25 µFD using Gene Pulser II (Bio-Rad). The CHO cells introduced with these DNAs by electroporation were added to CHO-S-SFMII medium (Invitrogen) containing 500 µg/mL

10  Geneticin and 1x HT (Invitrogen). A human Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-human Mpl"), monkey Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-monkey Mpl"), and mouse Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-mouse Mpl") were established through selection.

[0092]

15  1.3 Preparation of soluble human Mpl protein

To prepare soluble human Mpl protein, an expression system using insect Sf9 cells for production and secretion of the protein was constructed as described below.

A DNA construct encoding the extracellular region of human Mpl (Gln 26 to Trp 491) with a downstream FLAG tag was prepared. The construct was inserted into a pBACSurf-1

20  Transfer Plasmid (Novagen) between the *Pst*I and *Sma*I sites to prepare pBACSurf1-hMpl-FLAG. Then, Sf9 cells were transformed with 4 µg of pBACSurf1-hMpl-FLAG using the Bac-N-Blue Transfection Kit (Invitrogen). The culture supernatant was collected after three-day incubation. Recombinant virus was isolated by plaque assays. The prepared virus stock was used to infect Sf9 cells, and the culture supernatant was collected.

25  Soluble human Mpl protein was purified from the obtained culture supernatant as described below. The culture supernatant was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) for adsorption, and the adsorbed protein was then eluted with 50 mM Na-phosphate buffer (pH7.2) containing 0.01% (v/v) Tween20 and 500 mM NaCl. After the eluates were loaded onto a FLAG M2-Agarose (Sigma-Aldrich) for adsorption, the protein

30  adsorbed was eluted with 100 mM glycine-HCl buffer (pH3.5) containing 0.01% (v/v) Tween20. Immediately after elution, the fraction obtained was neutralized with 1 M Tris-Cl (pH8.0) and the buffer was exchanged with PBS (-) and 0.01% (v/v) Tween20 using PD-10 columns (Amersham Biosciences). The purified soluble Mpl protein was referred to as "shMpl-FLAG".

[0093]

35  1.4 Preparation of human Mpl-IgG Fc fusion protein

Human fusion protein Mpl-IgG Fc gene was prepared according to the method by

Bennett *et al.* (Bennett, B. D. *et al.*, J. Biol. Chem. (1991) 266: 23060-23067). A nucleotide sequence encoding the extracellular region of human Mpl (Gln 26 to Trp 491) was linked to a nucleotide sequence encoding the Fc region of human IgG-γ1 (a region downstream of Asp 216). A *Bst*EII sequence (amino acids: Val-Thr) was attached to the junction as a fusion linker between
5  these two regions. A 19-amino acid signal peptide derived form human IgG H chain variable region was used as the signal sequence. The resulting human fusion protein Mpl-IgG Fc gene was cloned into pCXND3 to construct pCXND3-hMpl-Fc.

The vector thus prepared (25 µg) was mixed with a PBS suspension of CHO-DG44 cells ($1 \times 10^7$ cells/mL) in Gene Pulser cuvettes. The mixture was then pulsed at 1.5 kV and 25 µFD
10  using Gene Pulser II (Bio-Rad). The CHO cells introduced with the DNA by electroporation were added to CHO-S-SFMII medium containing 500 µg/mL Geneticin and 1x HT (Invitrogen). shMPL-Fc-expressing CHO cell line (CHO-hMpl-Fc) was then established through selection.

Human Mpl-IgG Fc fusion protein was purified from the culture supernatant as described below.
15  The culture supernatant was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) for adsorption, and then the adsorbed protein were eluted with 50 mM Na-phosphate buffer (pH7.6) containing 0.01% (v/v) Tween20 and 1 M NaCl. After the eluates were loaded onto a HiTrap protein G HP column (Amersham Biosciences) for adsorption, the adsorbed protein was eluted with 0.1 M glycine-HCl buffer (pH2.7) containing 150 mM NaCl
20  and 0.01% (v/v) Tween20. Immediately after elution, the obtained fraction was neutralized with 1 M Tris-Cl (pH8.0) and the buffer was exchanged with PBS (-) and 0.01% (v/v) Tween20 using PD-10 columns (Amersham Biosciences). The purified soluble Mpl protein was referred to as "hMpl-Fc".

[0094]
25  1.5 Immunization with shMpl-FLAG and hybridoma selection

MRL/MpJUmmCrj-lpr/lpr mice (hereinafter abbreviated as "MRL/lpr mice"; purchased from Charles River, Japan) were immunized; the primary immunization was carried out at eight weeks of age. For every single mouse, an emulsion containing 100 µg of shMPL-FLAG combined with Freund's complete adjuvant (H37 Ra; Beckton Dickinson), was administered
30  subcutaneously as the primary injection. As a booster injection, an emulsion containing shMPL-FLAG (50 µg per mouse) combined with Freund's incomplete adjuvant (Beckton Dickinson) was administered subcutaneously. Three mice which have been immunized six times in total were subjected to a final injection of shMPL-FLAG (50 µg per mouse) through the caudal vein. Cell fusion was achieved by mixing the mouse myeloma P3-X63Ag8U1 cells
35  (P3U1; purchased from ATCC) and mouse splenocytes using polyethylene glycol 1500 (Roche Diagnostics). Hybridoma selection in HAT medium began the following day and culture

supernatants were obtained.    Screening was carried out by ELISA, using immunoplates immobilized with shMpl-FLAG or hMpl-Fc and the assayed cell growth activity of BaF3-hMpl as an index.    Positive clones were isolated as single clones by limiting dilution and then cultured in a large scale.    The culture supernatants were collected.

5        [0095]

1.6 Analyses of anti-human Mpl antibodies

Antibody concentrations were determined by carrying out a mouse IgG sandwich ELISA using goat anti-mouse IgG (gamma) (ZYMED) and alkaline phosphatase-goat anti-mouse IgG (gamma) (ZYMED), generating a calibration curve by GraphPad Prism

10    (GraphPad Software; USA), and calculating the antibody concentrations from the calibration curve.    Commercially available antibodies of the same isotype were used as standards.

[0096]

Antibody isotypes were determined by antigen-dependent ELISA using isotype-specific secondary antibodies.    hMpl-Fc was diluted to 1 μg/mL with a coating buffer (0.1 mM NaHCO$_3$,

15    pH9.6) containing 0.02% (w/v) NaN$_3$, and then added to ELISA plates.    The plates were incubated overnight at 4°C for coating.    The plates were blocked with a diluent buffer (50 mM Tris-HCl (pH8.1) containing 1 mM MgCl$_2$, 150 mM NaCl, 0.05% (v/v) Tween20, 0.02% (w/v) NaN$_3$, 1% (w/v) BSA).    After the addition of hybridoma culture supernatants, the plates were allowed to stand at room temperature for 1 hr.    After washing with a rinse buffer (0.05% (v/v)

20    Tween20 in PBS), alkaline phosphatase-labeled isotype-specific secondary antibodies were added to the plates.    Then, the plates were allowed to stand at room temperature for 1 hr. Color development was carried out using SIGMA104 (Sigma-Aldrich) diluted to 1 mg/mL with a substrate buffer (50 mM NaHCO$_3$, pH9.8) containing 10 mM MgCl$_2$, and absorbance was measured at 405 nm using Benchmark Plus (Bio-Rad).

25        [0097]

The binding activities of an antibody to shMpl-FLAG and hMPL-Fc were determined by ELISA.    ELISA plates were coated with 1 μg/mL of purified shMpl-FLAG or hMPL-Fc, and blocked with a diluent buffer.    Hybridoma culture supernatants were added to the plates, and the plates were allowed to stand at room temperature for 1 hr.    Then, alkaline phosphatase-labeled

30    anti-mouse IgG antibodies (Zymed) were added to the plates.    Color development was similarly carried out using the above method.    Following a one-hour coloring reaction at room temperature, absorbance was measured at 405 nm and EC$_{50}$ values were computed using GraphPad Prism.

[0098]

35        CHO-human Mpl cells and CHO-monkey Mpl cells were harvested, and suspended in FACS Buffer (1% FBS/ PBS) to a final concentration of 1 x 10$^6$ cells/mL.    The suspensions

were aliquoted into Multiscreen (Millipore) at 100 µl/well, and the culture supernatants were removed by centrifugation.    Culture supernatants diluted to 5 µg/mL were added to the plates and incubated on ice for 30 min.    The cells were washed once with FACS buffer, and incubated on ice for 30 min following the addition of an FITC-labeled anti-mouse IgG antibody (Beckman Coulter).    After incubation, the mixture was centrifuged at 500 rpm for 1 min.    The supernatants were removed, and then the cells were suspended in 400 µL of FACS buffer.    The samples were analyzed by flow cytometry using EPICS ELITE ESP (Beckman Coulter).    An analysis gate was set on the forward and side scatters of a histogram to include viable cell populations.

[0099]

Agonistic activities of an antibody were evaluated using BaF3-human Mpl and BaF3-monkey Mpl which proliferate in a TPO-dependent manner.    Cells of each cell line were suspended at $4 \times 10^5$ cells/ml in RPMI 1640/10% FBS (Invitrogen), and each suspension was aliquoted into a 96-well plate at 60µl/well.    A 40-µL aliquot of rhTPO (R&D) and hybridoma culture supernatants prepared at various concentrations was added into each well.    The plates were then incubated at 37°C under 5% $CO_2$ for 24 hr.    A 10-µL aliquot of the Cell Count Reagent SF (Nacalai Tesque) was added into each well.    After incubation for 2 hr, absorbance was measured at 450 nm (and at 655 nm as a control) using a Benchmark Plus.    $EC_{50}$ values were calculated using GraphPad Prism.

The above analysis yielded a total of 163 clones of mouse monoclonal antibodies that bind to human Mpl.

[0100]

1.7 Purification of anti-human Mpl antibodies

Anti-human Mpl antibodies were purified from hybridoma culture supernatants as described below.

After the culture supernatants were loaded onto HiTrap protein G HP columns (Amersham Biosciences) for adsorption, the antibodies were eluted with 0.1 M glycine-HCl (pH2.7).    Immediately after elution, the fractions were neutralized with 1 M Tris-Cl (pH9.0), dialyzed against PBS for one day, and the buffer was replaced.

[0101]

1.8 Determination of epitopes for the anti-human Mpl antibody VB22B

Since the anti-human Mpl antibody VB22B can be used for Western blotting, a GST-fusion protein containing a partial sequence of human Mpl was constructed for VB22B epitope analysis.    MG1 (Gln26 to Trp491) and MG2 (Gln26 to Leu274) regions were each amplified by PCR, and cloned into pGEX-4T-3 (Amersham Biosciences) to be expressed as GST fusion proteins.    The resulting plasmid DNAs were transformed into DH5α to give

transformants.    A final concentration of 1 mM IPTG was added to the transformants in their logarithmic growth phase to induce the expression of GST fusion proteins.    The bacterial cells were harvested after two hours of incubation.    The cells were lysed by sonication.    The lysates were centrifuged in XL-80 Ultracentrifuge (Beckman, Rotor 70.1Ti) at 35,000 rpm for 30 min.

5    The culture supernatants were removed, and then the fusion proteins were purified using GST Purification Modules (Amersham Biosciences).    The samples were separated by 10%-SDS-PAGE, and then transferred onto a PVDF membrane.    The membrane was Western blotted with the murine antibody VB22B.    VB22B was found to recognize both MG-1 and MG-2, indicating that the VB22B epitope is located in the (Gln26 to Leu274) region.

10        [0102]

Then, GST fusion proteins containing the respective regions of human Mpl: MG3 (Gln26 to Ala189), MG4 (Gln26 to Pro106), MG5 (Gln26 to Glu259), and MG6 (Gln26 to Gly245) were prepared and analyzed by Western blotting using the same procedure described above.    VB22B was found to recognize MG5 and MG6, but not MG3 and MG4.    This

15    suggests that the VB22B epitope is located within the (Ala189 to Gly245) region.    In addition, GST was fused with MG7 (Gln26 to Ala231) and MG8 (Gln26 to Pro217) to prepare GST fusion proteins.    VB22B recognized MG7 but not MG8, suggesting that the VB22B epitope is located in the (Gln217 to Ala231) region.    Furthermore, GST fusion protein containing MG10 (Gln213 to Ala231) was recognized by VB22B, suggesting that the VB22B epitope is located within the

20    limited region of 19 amino acids between Gln213 and Ala231.

[0103]

1.9 Kinetic analyses of the antigen-antibody reaction for anti-human Mpl antibody VB22B

Since the anti-human Mpl antibody VB22B binds to soluble recombinant Mpl, kinetic analyses of the antigen-antibody reaction between VB22B IgG and human Mpl-IgG Fc fusion

25    protein were carried out as described in Example 1.4.    The Sensor Chip CM5 (Biacore) was placed in Biacore 2000 (Biacore), and human Mpl-IgG Fc fusion protein was immobilized onto the chip by amine-coupling methods.    Then, 1.25 to 20 µg/mL of VB22B IgG solution was prepared using HBS-EP Buffer (Biacore), and injected over the chip surface for 2 min to reveal the binding region.    Then, HBS-EP Buffer was injected over the chip surface for 2 min to reveal

30    the dissociation region.    VB22B IgG bound to the human Mpl-IgG Fc fusion protein on the sensor chip was removed by injecting 10 mM NaOH over the sensor chip for 15 sec, and the chip was recovered.    HBS-EP Buffer was used as the running buffer, and the flow rate was 20 µL/min.    Using the BIAevaluation Version 3.1 (Biacore) software, the reaction rate constant at each concentration was calculated from the sensorgrams.    The dissociation constant (KD) for

35    VB22B IgG was determined to be $1.67 \pm 0.713 \times 10^{-9}$ M.

[0104]

[Example 2]    Preparation of single-chain anti-human Mpl antibodies

Among the prepared anti-human Mpl antibodies, 23 types of antibodies, which exhibit higher binding activities and agonistic activities, were selected to construct expression systems for single-chain antibodies using genetic engineering techniques.    An exemplary method for constructing a single-chain antibody derived from the anti-human Mpl antibody VB22B is described below.

[0105]

2.1 Cloning of the anti-human Mpl antibody variable region

The variable region was amplified by RT-PCR using total RNA extracted from hybridomas producing anti-human Mpl antibodies.    Total RNA was extracted from 1 x 10$^7$ hybridoma cells using the RNeasy Plant Mini Kit (QIAGEN).

[0106]

A 5'-terminal fragment of the gene was amplified from 1 μg of total RNA by the SMART RACE cDNA Amplification Kit (Clontech), using a synthetic oligonucleotide MHC-IgG2b (SEQ ID NO: 166) complementary to mouse IgG2b constant region or a synthetic oligonucleotide kappa (SEQ ID NO: 167) complementary to mouse κ chain constant region. Reverse transcription was carried out at 42°C for 1.5 hr.

[0107]

The composition of the PCR reaction solution (50 μL in total) is shown below.

| | |
|---|---|
| 10x Advantage 2 PCR Buffer (Clontech) | 5 μL |
| 10x Universal Primer A Mix (Clontech) | 5 μL |
| dNTPs (dATP, dGTP, dCTP, and dTTP) (Clontech) | 0.2 mM |
| Advantage 2 Polymerase Mix (Clontech) | 1 μL |
| Reverse transcription product | 2.5 μL |
| Synthetic oligonucleotide, MHC-IgG2b or kappa | 10 pmol |

[0108]

The PCR reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 5 sec and 72°C for 3 min;

five cycles of 94°C for 5 sec, 70°C for 10 sec, and 72°C for 3 min;

25 cycles of 94°C for 5 sec, 68°C for 10 sec, and 72°C for 3 min;

and final extension was at 72°C for 7 min.

[0109]

The PCR products were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and cloned into a pGEM-T Easy Vector (Promega).    The nucleotide sequence was then determined using the ABI 3700 DNA Analyzer (Perkin Elmer).

[0110]

The nucleotide sequence of cloned VB22B H chain variable region (hereinafter abbreviated as "VB22B-VH") is shown in SEQ ID NO: 117, and its amino acid sequence is shown in SEQ ID NO: 118.   The nucleotide sequence of the L chain variable region (hereinafter abbreviated as "VB22B-VL") is shown in SEQ ID NO: 119, and its amino acid sequence is shown in SEQ ID NO: 120.

[0111]

2.2 Preparation of expression vectors for anti-human Mpl diabodies

The gene encoding VB22B single-chain Fv (hereinafter abbreviated as "VB22B diabody") containing a five-amino acid linker sequence was constructed, by linking a nucleotide sequence encoding a (Gly4Ser)₁ linker to the VB22B-VH-encoding gene at its 3' end and to the VB22B-VL-encoding gene at its 5' end; both of which have been amplified by PCR.

[0112]

The VB22B-VH forward primer, 70·115HF, (SEQ ID NO: 168) was designed to contain an EcoRI site.   The VB22B-VH reverse primer, 33·115HR, (SEQ ID NO: 169) was designed to hybridize to a DNA encoding the C terminus of VB22B-VH, and to have a nucleotide sequence encoding the (Gly4Ser)₁ linker and a nucleotide sequence hybridizing to the DNA encoding the N terminus of VB22B-VL.   The VB22B-VL forward primer, 33·115LF, (SEQ ID NO: 170) was designed to have a nucleotide sequence encoding the N terminus of VB22B-VL, a nucleotide sequence encoding the (Gly4Ser)₁ linker, and a nucleotide sequence encoding the C terminus of VB22B-VH.   The VB22B-VL reverse primer, 33·115LR, (SEQ ID NO: 171) was designed to hybridize to a DNA encoding the C terminus of VB22B-VL and to have a nucleotide sequence encoding a FLAG tag (Asp Tyr Lys Asp Asp Asp Asp Lys/SEQ ID NO: 172) and a NotI site.

[0113]

In the first round of PCR, two PCR products: one containing VB22B-VH and a linker sequence, and the other containing VB22B-VL and the identical linker sequence, were synthesized by the procedure described below.

The composition of the PCR reaction solution (50 µL in total) is shown below.

| | |
|---|---|
| 10x PCR Buffer (TaKaRa) | 5 µL |
| dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa) | 0.4 mM |
| DNA polymerase TaKaRa Ex Taq (TaKaRa) | 2.5 units |
| pGEM-T Easy vector comprising VB22B-VH or VB22B-VL gene | 10 ng |
| Synthetic oligonucleotides, 70·115HF and 33·115HR, or 33·115LF and 33·115LR | 10 pmol |

[0114]

The PCR reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of: 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0115]

After the PCR products of about 400 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), the second-round PCR was carried out using aliquots of the respective PCR products according to the protocol described below.

The composition of the PCR reaction solution (50 µL in total) is shown below.

| | |
|---|---|
| 10x PCR Buffer (TaKaRa) | 5 µL |
| dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa) | 0.4 mM |
| DNA polymerase TaKaRa Ex Taq (TaKaRa) | 2.5 unit |
| First-round PCR products (two types) | 1 µL |
| Synthetic oligonucleotides, 70·115HF and 33·115LR | 10 pmol |

[0116]

The reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0117]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then digested with *Eco*RI and *Not*I (both from TaKaRa). The resulting DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN), and then cloned into pCXND3 to prepare pCXND3-VB22B db.

[0118]

2.3 Preparation of expression vectors for anti-human Mpl antibody sc(Fv)$_2$

To prepare expression plasmids for the modified antibody [sc(Fv)$_2$] comprising two units of H chain variable region and two units of L chain variable region derived from VB22B, the above-described pCXND3-VB22B db was modified by PCR using the procedure shown below. The process for constructing the sc(Fv)$_2$ gene is illustrated in Fig. 1.

[0119]

First, PCR method was carried out to amplify (a) the VB22B-VH-encoding gene in which a nucleotide sequence encoding a 15-amino acid linker (Gly$_4$Ser)$_3$ was added to its 3' end;

and (b) the VB22B-VL-encoding gene containing the identical linker nucleotide sequence added to its 5' end.   The desired construct was prepared by linking these amplified genes.   Three new primers were designed in this construction process.   The VB22B-VH forward primer, VB22B-fpvu, (primer A; SEQ ID NO: 173) was designed to have an *Eco*RI site at its 5' end and to convert Gln22 and Leu23 of VB22B db into a *Pvu*II site.   The VB22B-VH reverse primer, sc-rL15, (primer B; SEQ ID NO: 174) was designed to hybridize to a DNA encoding the C terminus of VB22B-VH, and to have a nucleotide sequence encoding the (Gly4Ser)$_3$ linker, as well as a nucleotide sequence hybridizing to a DNA encoding the N terminus of VB22B-VL. The VB22B-VL forward primer, sc-fL15, (primer C; SEQ ID NO: 175) was designed to have a nucleotide sequence encoding the N terminus of VB22B-VL, a nucleotide sequence encoding the (Gly$_4$Ser)$_3$ linker, and a nucleotide sequence encoding the C terminus of VB22B-VH.

[0120]

In the first-round PCR, two PCR products: one comprising VB22B-VH and a linker sequence, and the other comprising VB22B-VL and the identical linker sequence, were synthesized by the procedure described below.

The composition of the PCR reaction solution (50 μL in total) is shown below.

| | |
|---|---|
| 10x PCR Buffer (TaKaRa) | 5 μL |
| dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa) | 0.4 mM |
| DNA polymerase TaKaRa Ex Taq (TaKaRa) | 2.5 units |
| pCXND3-VB22B db | 10 ng |
| Synthetic oligonucleotides, VB22B-fpvu, sc-rL15 or sc-fL15, and 33·115LR (primer D) | 10 pmol |

[0121]

The reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0122]

After the PCR products of about 400 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), the second-round PCR was carried out using aliquots of the respective PCR products according to the protocol described below.

The composition of the PCR reaction solution (50 μL in total) is shown below.

| | |
|---|---|
| 10x PCR Buffer (TaKaRa) | 5 μL |
| dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa) | 0.4 mM |

| DNA polymerase TaKaRa Ex Taq (TaKaRa) | 2.5 units |
| First-round PCR product (two types) | 1 μL |
| Synthetic oligonucleotide, 70·115HF and 33·115LR | 10 pmol |

[0123]

The reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0124]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick

Gel Extraction Kit (QIAGEN), and then digested with *EcoRI* and *NotI* (both from TaKaRa).

The resulting DNA fragments were purified using the QIAquick PCR Purification Kit

(QIAGEN), and then cloned into pBacPAK9 (Clontech) to construct pBacPAK9-scVB22B.

[0125]

A fragment to be inserted into the *PvuII* site of pBacPAK9-scVB22B was prepared.

Specifically, the fragment has a *PvuII* recognition site at both ends and a nucleotide sequence, in

which a gene encoding the VB22B-VH N-terminus is linked, via a (Gly₄Ser)₃ linker-encoding

nucleotide sequence, to a gene encoding the amino acid sequence of an N terminus-deleted

VB22B-VH linked to VB22B-VL via the (Gly₄Ser)₃ linker.   Two primers were newly designed

to prepare the fragment by PCR.   The forward primer for the fragment of interest, Fv2-f (primer

E; SEQ ID NO: 176), was designed to have a *PvuII* site at its 5' end and a VB22B-VH 5'-end

sequence.   The reverse primer for the fragment of interest, Fv2-r (primer F; SEQ ID NO: 177),

was designed to hybridize to a DNA encoding the C terminus of VB22B-VL, and to have a *PvuII*

site, a nucleotide sequence encoding the (Gly₄Ser)₃ linker, and a nucleotide sequence hybridizing

to a DNA encoding the N terminus of VB22B-VH.   PCR was carried out using

pBacPAK9-scVB22B as a template as described below.

[0126]

The composition of the PCR reaction solution (50 μL in total) is shown below.

| 10x PCR Buffer (TaKaRa) | 5 μL |
| dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa) | 0.4 mM |
| DNA polymerase TaKaRa Ex Taq (TaKaRa) | 2.5 units |
| pBacPAK9-scVB22B | 10 μg |
| Synthetic oligonucleotide, Fv2-f and Fv2-r | 10 pmol |

[0127]

The reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0128]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then cloned into the pGEM-T Easy Vector (Promega). After sequencing, the plasmid was digested with *Pvu*II (TaKaRa), and the fragment of interest was recovered. The recovered fragment was ligated to pBacPAK9-scVB22B pre-digested with *Pvu*II (TaKaRa) to construct pBacPAK9-VB22B sc(Fv)$_2$. After the resulting vector was digested with *Eco*RI and *Not*I (both from TaKaRa), the fragment of about 1,800 bp was purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN). The fragment was then cloned into a pCXND3 expression vector to construct pCXND3-VB22B sc(Fv)$_2$.

[0129]

2.4 Expression of single-chain anti-human Mpl antibody in animal cells

A cell line stably expressing the single-chain antibody was prepared from CHO-DG44 cells as described below. Gene transfer was achieved by electroporation using a Gene Pulser II (Bio-Rad). An expression vector (25 μg) and 0.75 mL of CHO-DG44 cells suspended in PBS (1 x 10$^7$ cells/mL) were mixed. The resulting mixture was cooled on ice for 10 min, transferred into a cuvette, and pulsed at 1.5-kV and 25 μFD. After a ten-minute restoration period at room temperature, the electroporated cells were plated in CHO-S-SFMII medium (Invitrogen) containing 500 μg/mL Geneticin (Invitrogen). CHO cell lines expressing the single-chain antibody were established through selection. A cell line stably expressing VB22B sc(Fv)$_2$ and its culture supernatants were obtained by this method.

[0130]

The transient expression of the single-chain antibody was achieved using COS7 cells as described below. An expression vector (10 μg) and 0.75 mL of CHO-DG44 cells suspended in PBS (1 x 10$^7$ cells/mL) were mixed. The resulting mixture was cooled on ice for 10 min, transferred into a cuvette, and then pulsed at 1.5-kV and 25 μFD. After a ten-minute restoration period at room temperature, the electroporated cells were plated in DMEM/10% FBS medium (Invitrogen). The cells were incubated overnight and then washed with PBS. CHO-S-SFMII medium was added and the cells were cultured for about three days. The culture supernatants for preparing the VB22B diabody were thus prepared.

[0131]

2.5 Quantitation of single-chain anti-human Mpl antibodies in culture supernatants

The culture supernatant concentration of the single-chain anti-human Mpl antibody transiently expressed in COS cells was determined using surface plasmon resonance.    A sensor chip CM5 (Biacore) was placed in Biacore 2000 (Biacore).    ANTI-FLAG® M2 Monoclonal

5    Antibody (Sigma-Aldrich) was immobilized onto the chip.    An appropriate concentration of sample was injected over the chip surface at a flow rate of 5 mL/sec, and 50 mM diethylamine was used to dissociate the bound antibody.    Changes in the mass during sample injection were recorded, and the sample concentration was calculated from the calibration curve prepared using the mass changes of a standard sample.    db12E10 (see WO 02/33073 and WO 02/33072) was

10   used as the diabody standard, and 12E10 sc(Fv)$_2$ which has the same gene structure as that of sc(Fv)$_2$ was used as the sc(Fv)$_2$ standard.

[0132]

2.6 Purification of anti-human Mpl single-chain antibodies

The culture supernatants of VB22B diabody-expressing COS7 cells or CHO cells was

15   loaded onto an Anti-Flag M2 Affinity Gel (Sigma-Aldrich) column equilibrated with a 50 mM Tris-HCl buffer (pH7.4) containing 150 mM NaCl and 0.05% Tween20.    The absorbed antibodies were eluted with 100 mM glycine-HCl (pH3.5).    The fractions eluted were immediately neutralized with 1 M Tris-HCl (pH8.0), and loaded onto a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column for gel filtration chromatography.    PBS/0.01%

20   Tween20 was used in the gel filtration chromatography.

[0133]

VB22B sc(Fv)$_2$ was purified from the culture supernatants of VB22B sc(Fv)$_2$-expressing COS7 cells or CHO cells under the same conditions used for purifying the diabodies.    A large-scale preparation of VB22B sc(Fv)$_2$ was prepared by loading the CHO cell culture

25   supernatants onto a Macro-Prep Ceramic Hydroxyapatite Type I (Bio-Rad) column equilibrated with a 20 mM phosphate buffer (pH6.8), and eluting the VB22B sc(Fv)$_2$ in a stepwise manner with 250 mM phosphate buffer (pH6.8).    The eluted fraction was concentrated on an ultrafilter, and then fractionated by gel filtration chromatography using a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column, and a fraction corresponding to the molecular weight range of

30   about 40 kD to 70 kD was obtained.    The fraction was loaded onto an Anti-Flag M2 Affinity Gel column equilibrated with a 50 mM Tris-HCl buffer (pH7.4) containing 150 mM NaCl and 0.05% Tween20.    The absorbed antibody was eluted with 100 mM glycine-HCl (pH3.5).    The eluted fraction was immediately neutralized with 1 M Tris-HCl (pH8.0), and loaded onto a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column for gel filtration

35   chromatography.    20 mM acetate (pH6.0) containing 150 mM NaCl and 0.01% Tween80 was used in the gel filtration chromatography.

[0134]

2.7 Binding activity analyses of single-chain anti-human Mpl antibodies by flow cytometry

CHO-human Mpl, CHO-monkey Mpl, and CHO-mouse Mpl cells were recovered and suspended in FACS buffer (1% FBS/PBS) to a final concentration of 1 x $10^6$ cells/mL. Cell suspensions were aliquoted at 100-μL/well into the Multiscreen-HV Filter Plates (Millipore). After centrifugation, the supernatant was removed. An appropriate concentration of diabody or sc(Fv)$_2$ was added into each well and incubated on ice for 30 min. The cells were washed once with 200 μL of FACS buffer, and incubated on ice for 30 min following the addition of 10 μg/mL ANTI-FLAG® M2 Monoclonal Antibody (Sigma-Aldrich). The cells were then washed once with 200 μL of FACS buffer, and a 100x-diluted FITC-labeled anti-mouse IgG antibody (Beckman Coulter) was added to the plate. The plate was incubated on ice for 30 min. After centrifugation, the supernatant was removed. The cells were suspended in 400 μL of FACS Buffer, and then analyzed by flow cytometry using EPICS ELITE ESP (Beckman Coulter). An analysis gate was set on the forward and side scatters of a histogram to include viable cell populations.

[0135]

The binding activity of the purified VB22B sc(Fv)$_2$ to various Mpl molecules expressed in CHO cells was determined (Fig. 2). VB22B sc(Fv)$_2$ was found to specifically bind to CHO-human Mpl and CHO-monkey Mpl but not to the host cell CHO or CHO-mouse Mpl. This binding characteristic of VB22B sc(Fv)$_2$ is comparable to those of VB22B IgG, indicating that the antibody binding site remains unaltered by reduction of molecular weight.

[0136]

2.8 Analyses of TPO-like agonistic activity for single-chain anti-human Mpl antibodies

TPO-like agonistic activity was assessed using BaF3-human Mpls or BaF3-monkey Mpls that proliferate in a TPO-dependent manner.

[0137]

Cells from each cell line were washed twice with RPMI 1640/1% FBS (fetal bovine serum) (Invitrogen), and then suspended in RPMI 1640/10% FBS to a concentration of 4 x $10^5$ cells/mL. Cell suspensions were aliquoted at 60-μL/well into a 96-well plate. Various concentrations of rhTPO (R&D) and COS7 culture supernatants or purified samples were prepared, and a 40-μL aliquot was added into each well. The plates were then incubated at 37°C under 5% $CO_2$ for 24 hr. Immediately after a 10-μL aliquot of WST-8 reagent (Cell Count Reagent SF; Nacalai Tesque) was added into each well, absorbance was measured at 450 nm (and at 655 nm as a control) using Benchmark Plus. After two hours of incubation, absorbance was again measured at 450 nm (and at 655 nm as a control). The WST-8 reagent changes colors at 450 nm in a color reaction that reflects the viable cell count. The TPO-like

agonistic activity was assessed using the change in absorbance during the two-hour incubation as an index.   $EC_{50}$ values were computed using GraphPad Prism.

[0138]

TPO-like agonistic activity was assayed using the human leukemia cell line M-07e (purchased from DSMZ) which proliferates TPO-dependently.   M-07e cells were washed twice with RPMI 1640/1% FBS, and then suspended in RPMI 1640/10% FBS to a concentration of 5 x $10^5$ cells/mL.   The resulting cell suspension was aliquoted at 50-μL/well into a 96-well plate. Various concentrations of rhTPO and COS7 culture supernatants or purified samples were prepared, and a 50-μL aliquot was added into each well.   The plates were then incubated at 37°C under 5% $CO_2$ for 48 hr.   Immediately after a 10-μL aliquot of WST-8 reagent (Cell Count Reagent SF; Nacalai Tesque) was added to each well, absorbance of was measured at 450 nm (and at 655 nm as a control) using a Benchmark Plus.   After four hours of incubation, absorbance was again measured at 450 nm (and at 655 nm as a control).   The TPO-like agonistic activity was assayed using the change in absorbance during the four-hour incubation as an index.

[0139]

Purified VB22B IgG, VB22B diabody, and VB22B sc(Fv)₂ were assayed for their TPO-like agonistic activities using BaF3-human Mpl, BaF3-monkey Mpl, and M-07e.   The results are shown in Figures 3, 4, and 5, respectively.   The presence of bivalent antigen-binding domains in a single antibody molecule is essential for its agonistic activity.   The distance and angle between two antigen-binding domains can also be important factors (see WO 02/33073 and WO 02/33072).   Similar results were obtained for the newly isolated anti-human Mpl antibodies.   Specifically, the agonistic activities of VB22B diabody and VB22B sc(Fv)₂ ($EC_{50}$ = 61 pM and 27 pM in BaF-human Mpl, respectively) were higher than that of VB22B IgG ($EC_{50}$ > 30 nM in BaF-human Mpl), and were equivalent to or higher than that of the naturally-occurring human TPO ligand ($EC_{50}$ = 76 pM in BaF-human Mpl).   The VB22B diabody activity was lower than that of VB22B sc(Fv)₂.   This suggests that the structure of a single-chain antibody is greatly altered by its molecular shape and the length of the linker sequence, which in turn changes the agonistic activity.   Sixteen types of the single-chain anti-human Mpl antibodies were obtained, each exhibiting a high agonistic activity.   The amino acid sequences of the H chain and L chain variable regions of the representative antibodies are shown in Figures 6 and 7, respectively.

[0140]

2.9 Humanization of single-chain anti-human Mpl antibody

Antibody sequence data for the humanization of VB22B sc(Fv)₂ were obtained from the Kabat Database (ftp://ftp.ebi.ac.uk/pub/databases/kabat/), and homology searches were carried

out independently for the H chain variable region and the L chain variable region.  As a result, the H chain variable region was found to be highly homologous to DN13 (Smithson S. L. *et al.*, Mol Immunol. (1999) 36: 113-124).  The L chain variable region was found to be highly homologous to ToP027 (Hougs L. *et al.*, J. Immunol. (1999) 162: 224-237).  Humanized antibodies were prepared by inserting a complementarity-determining region (hereinafter abbreviated as "CDR") into the framework regions (hereinafter abbreviated as "FR") of the above antibodies.

[0141]

Specifically, synthetic oligo-DNAs of approximately 50 nucleotides in length were designed as to make 20 of these nucleotides available for hybridization, and the synthetic oligo-DNAs were assembled by PCR to prepare genes that encode the respective variable regions.  Using the resulting genes, sc(Fv)$_2$ was similarly prepared by the method described in Example 2.3.  The respective DNAs were cloned into a pCXND3expression vector to construct expression vectors pCXND3-hVB22B sc(Fv)$_2$.  The nucleotide sequence and the amino acid sequence of hVB22B sc(Fv)$_2$ in plasmid are shown in SEQ ID NO:1 and SEQ ID NO: 2.

[0142]

[Example 3]    Preparation of anti-Mpl diabodies by the AGS method

Anti-Mpl diabodies having agonistic activity were prepared by an Autocrine Growth Selection (AGS) method (see, WO 03/91424).

[0143]

3.1 Construction of a retrovirus library

Spleens were isolated from MRL/lpr mice immunized with shMPL-Flag by the method described in Example 1.5, and homogenized in TRIZOL Reagent (Invitrogen) using a Dounce homogenizer.  After chloroform addition, the homogenized sample was shaken vigorously, the aqueous phase was removed and total RNA was extracted by isopropanol precipitation.  mRNA was purified using a PolyATract System 1000 (Promega).  Reverse transcription of 2.5 μg mRNA was carried out at 42°C for 50 min using the Superscript First strand synthesis system for RT-PCR (Invitrogen) and the included oligo-dT primers to prepare cDNA.

[0144]

The composition of the PCR reaction solution (250 μL) is shown below.

| | |
|---|---|
| 10x KOD Plus Buffer (Toyobo) | 25 μL |
| 2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (Toyobo) | 25 μL |
| 2.5 mM MgSO$_4$ (Toyobo) | 10 μL |
| KOD Plus (Toyobo) | 7.5 μL |
| Reverse transcription products | 25 μL |
| Mixed primers complementary to H chain or L chain variable region | 500 pmol |

[0145]

The reaction conditions were:

98°C (initial temperature) for 3 min;

32 cycles of 98°C for 20 sec, 58°C for 20 sec, and 72°C for 30 sec;

and final extension was at 72°C for 6 min.

[0146]

The H chain primer mix contained HS1 to HS19 (SEQ ID NOs: 178 to 196) and HA1 to HA4 (SEQ ID NOs: 197 to 200), which were mixed at the indicated ratios next to the sequence names in Table 1.   The L chain primer mix contained LS1 to LS17 (SEQ ID NOs: 201 to 217), LSlambda (SEQ ID NO: 218), LA1 to LA5 (SEQ ID NOs: 219 to 222), and LAlambda (SEQ ID NO: 223).   The respective PCR products were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN).   The H chain and L chain variable regions were linked via the (Gly$_4$Ser)$_1$ linker sequence by PCR using sc-S (SEQ ID NO: 224) and sc-AS (SEQ ID NO: 225) as described below.

[0147]

The composition of the PCR reaction solution (100 μL in total) is shown below.

| | |
|---|---|
| 10x KOD Plus Buffer (Toyobo) | 10 μL |
| 2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (Toyobo) | 10 μL |
| 2.5 mM MgSO$_4$ (Toyobo) | 4 μL |
| KOD Plus (Toyobo) | 2 μL |
| Fragment of H chain variable region | 4 μL |
| Fragment of L chain variable region | 4 μL |

The first-round PCR conditions were:

94°C (initial temperature) for 3 min; and

seven cycles of 94°C for 1 min and 63°C for 4 min.

Then, sc-S and sc-AS (25 pmol each) were added to the first-round products.

[0148]

The second-round PCR conditions were:

30 cycles of 94°C for 30 sec, 55°C for 2 min, and 72°C for 2 min;

and final extension was at 72°C for 6 min.

[0149]

The resulting product with an *Sfi*I restriction site at both ends was purified using the QIAquick PCR Purification Kit (QIAGEN), and incubated with the *Sfi*I restriction enzyme (TaKaRa) overnight at 50°C.   The PCR product purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN) was inserted into the *Sfi*I site of the viral vector pMX/IL3ssGFPHis.

[0150]

The resulting plasmid was constructed by inserting a GFP gene, which has an *Eco*RI site, mouse IL-3 signal sequence and *Sfi*I site at its 5' end; and an *Sfi*I site, His tag sequence, termination codon, and *Not*I site at its 3' end, between the *Eco*RI and *Not*I sites on the pMX viral vector (Onishi, M. *et al.*, Mol. Cell. Biol. 18: 3871-3879).    The plasmid was introduced into the ElectroMAX DH10B T1 phage resistant cells (Invitrogen) by electroporation (settings: 2.5 kV, 25 μF, and 100Ω) using a Gene Pulser II (Bio-Rad).    The cells were plated onto an LB-Agar plate containing 100 μg/mL ampicillin.    After overnight incubation, 1 x 10$^7$ colonies were obtained.    Colonies were recovered from the plate and plasmids were then extracted using the QIAGEN Plasmid Maxi Kit (QIAGEN).

[0151]

[Table 1]

```
SEQ ID NO:178 (HS1(4))   GCCCAGCCGGCCATGGCGGAKGTRMAGCTTCAGGAGTC
SEQ ID NO:179 (HS2(4))   GCCCAGCCGGCCATGGCGGAGGTBCAGCTBCAGCAGTC
SEQ ID NO:180 (HS3(3))   GCCCAGCCGGCCATGGCGCAGGTGCAGCTGAAGSASTC
SEQ ID NO:181 (HS4(4))   GCCCAGCCGGCCATGGCGGAGGTCCARCTGCAACARTC
SEQ ID NO:182 (HS5(7))   GCCCAGCCGGCCATGGCGCAGGTYCAGCTBCAGCARTC
SEQ ID NO:183 (HS6(2))   GCCCAGCCGGCCATGGCGCAGGTYCARCTGCAGCAGTC
SEQ ID NO:184 (HS7(1))   GCCCAGCCGGCCATGGCGCAGGTCCACGTGAAGCAGTC
SEQ ID NO:185 (HS8(2))   GCCCAGCCGGCCATGGCGGAGGTGAASSTGGTGGAATC
SEQ ID NO:186 (HS9(5))   GCCCAGCCGGCCATGGCGGAVGTGAWGYTGGTGGAGTC
SEQ ID NO:187 (HS10(2))  GCCCAGCCGGCCATGGCGGAGGTGCAGSKGGTGGAGTC
SEQ ID NO:188 (HS11(2))  GCCCAGCCGGCCATGGCGGAKGTGCAMCTGGTGGAGTC
SEQ ID NO:189 (HS12(2))  GCCCAGCCGGCCATGGCGGAGGTGAAGCTGATGGARTC
SEQ ID NO:190 (HS13(1))  GCCCAGCCGGCCATGGCGGAGGTGCARCTTGTTGAGTC
SEQ ID NO:191 (HS14(2))  GCCCAGCCGGCCATGGCGGARGTRAAGCTTCTCGAGTC
SEQ ID NO:192 (HS15(2))  GCCCAGCCGGCCATGGCGGAAGTGAARSTTGAGGAGTC
SEQ ID NO:193 (HS16(5))  GCCCAGCCGGCCATGGCGCAGGTTACTCTRAAAGWGTSTG
SEQ ID NO:194 (HS17(3.5)) GCCCAGCCGGCCATGGCGCAGGTCCAACTVCAGCARCC
SEQ ID NO:195 (HS18(0.7)) GCCCAGCCGGCCATGGCGGATGTGAACTTGGAAGTGTC
SEQ ID NO:196 (HS19(0.7)) GCCCAGCCGGCCATGGCGGAGGTGAAGGTCATCGAGTC
SEQ ID NO:197 (HA1(1))   GGAGCCGCCGCCGCCCGAGGAAACGGTGACCGTGGT
SEQ ID NO:198 (HA2(1))   GGAGCCGCCGCCGCCCGAGGAGACTGTGAGAGTGGT
SEQ ID NO:199 (HA3(1))   GGAGCCGCCGCCGCCCGCAGAGACAGTGACCAGAGT
SEQ ID NO:200 (HA4(1))   GGAGCCGCCGCCGCCCGAGGAGACGGTGACTGAGGT
SEQ ID NO:201 (LS1(1))   GGCGGCGGCGGCTCCGAYATCCAGCTGACTCAGCC
SEQ ID NO:202 (LS2(2))   GGCGGCGGCGGCTCCGAYATTGTTCTCWCCCAGTC
SEQ ID NO:203 (LS3(5))   GGCGGCGGCGGCTCCGAYATTGTGMTMACTCAGTC
SEQ ID NO:204 (LS4(3.5)) GGCGGCGGCGGCTCCGAYATTGTGYTRACACAGTC
SEQ ID NO:205 (LS5(4))   GGCGGCGGCGGCTCCGAYATTGTRATGACMCAGTC
SEQ ID NO:206 (LS6(7))   GGCGGCGGCGGCTCCGAYATTMAGATRAMCCAGTC
SEQ ID NO:207 (LS7(6))   GGCGGCGGCGGCTCCGAYATTCAGATGAYDCAGTC
SEQ ID NO:208 (LS8(1.5)) GGCGGCGGCGGCTCCGAYATYCAGATGACACAGAC
SEQ ID NO:209 (LS9(2))   GGCGGCGGCGGCTCCGAYATTGTTCTCAWCCAGTC
SEQ ID NO:210 (LS10(3.5)) GGCGGCGGCGGCTCCGAYATTGWGCTSACCCAATC
SEQ ID NO:211 (LS11(8))  GGCGGCGGCGGCTCCGAYATTSTRATGACCCARTC
SEQ ID NO:212 (LS12(8))  GGCGGCGGCGGCTCCGAYRTTKTGATGACCCARAC
SEQ ID NO:213 (LS13(6))  GGCGGCGGCGGCTCCGAYATTGTGATGACBCAGKC
SEQ ID NO:214 (LS14(2))  GGCGGCGGCGGCTCCGAYATTGTGATAACYCAGGA
SEQ ID NO:215 (LS15(2))  GGCGGCGGCGGCTCCGAYATTGTGATGACCCAGWT
SEQ ID NO:216 (LS16(1))  GGCGGCGGCGGCTCCGAYATTGTGATGACACAACC
SEQ ID NO:217 (LS17(1))  GGCGGCGGCGGCTCCGAYATTTTGCTGACTCAGTC
SEQ ID NO:218 (LSlambda(1)) GGCGGCGGCGGCTCCGATGCTGTTGTGACTCAGGAATC
SEQ ID NO:219 (LA1(4))   GGAATTCGGCCCCCGAGGCCTTGATTTCCAGCTTGG
SEQ ID NO:220 (LA2(4))   GGAATTCGGCCCCCGAGGCCTTTATTTCCAGCTTGG
SEQ ID NO:221 (LA4(4))   GGAATTCGGCCCCCGAGGCCTTTATTTCCAACTTTG
SEQ ID NO:222 (LA5(4))   GGAATTCGGCCCCCGAGGCCTTCAGCTCCAGCTTGG
SEQ ID NO:223 (LAlambda(1)) GGAATTCGGCCCCCGAGGCCCCTAGGACAGTCAGTTTGG
```

[0152]

3.2 Establishment of autonomously replicating cell lines by the AGS method

The resulting library was transfected into a packaging cell, Pt-E, (Morita, S. *et al.*, Gene therapy 7: 1063-1066) using FuGENE 6 (Roche Diagnostics). Specifically, Pt-E was plated onto 6-cm dishes and cultured in DMEM/10% FBS (Invitrogen). A mixture of FuGENE 6 and the library was added to the plate the following day. The culture medium was exchanged the next day, and the culture supernatant was collected 24 hours after that. 10 µg/mL polybrene (Hexadimethrine Bromide; Sigma) and 2 ng/mL mIL-3 were added to the culture supernatant containing recombinant virus particles. The viral solution was used to infect the BaF-monkey Mpl target cells. The cells were washed with PBS the following day, and suspended in RPMI 1640/10% FBS without mIL-3. The suspension was plated onto a 96-well plate at a cell density of 1,000 cells/well. Autonomously replicating cell lines (AB317 and AB324) were obtained after seven days of incubation. Genomic DNAs were extracted from these cells using a DNeasy Tissue Kit (QIAGEN), and the antibody genes were amplified by PCR.

[0153]

The composition of the PCR reaction solution (50 µL in total) is shown below.

| | |
|---|---|
| 10x LA Taq Buffer (TaKaRa) | 5 µL |
| 2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa) | 5 µL |
| 2.5 mM MgCl₄ (TaKaRa) | 5 µL |
| TaKaRa LA Taq (TaKaRa) | 0.5 µL |
| Genomic DNA | 0.5 µg |
| AGSdbS1 (SEQ ID NO: 226) and AGSdbA1 (SEQ ID NO: 227) | 25 pmol |

[0154]

The reaction conditions were:

94°C (initial temperature) for 1 min;

30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 70°C for 1 min;

and final extension was at 72°C for 6 min.

[0155]

The nucleotide sequence and the amino acid sequence of the H chain of cloned AB317 are shown in SEQ ID NOs: 154 and 155. The nucleotide sequence and the amino acid sequence of AB317 L chain are shown in SEQ ID NOs: 156 and 157. The nucleotide sequence and the amino acid sequence of AB324 H chain are shown in SEQ ID NOs: 158 and 159. The nucleotide sequence and the amino acid sequence of AB324 L chain are shown in SEQ ID NOs: 160 and 161.

[0156]

3.3 Activity assays of the diabodies obtained by AGS method

Each of the anti-Mpl diabodies obtained above was inserted into the pCXND3 expression vector.    The PCR primers used are a synthetic oligonucleotide complementary to the 5' end of the diabody and containing an *Eco*RI site, and a synthetic oligonucleotide complementary to the nucleotide sequence of the 3' end of the diabody and containing a FLAG tag and a *Not*I site.    The PCR product thus obtained was inserted into pCXND3 between the *Eco*RI and *Not*I sites.    The diabody was expressed transiently in COS7 cells by the method described in Example 2.4.    The culture supernatant was removed and the activity of the diabody was evaluated.

[0157]

The binding activities of the diabodies were assessed by flow cytometry using CHO cells that express Mpl derived from various species (Fig. 8).    AB317 was proven to bind to CHO-mouse Mpl.

[0158]

The TPO-like agonistic activities of the diabodies were evaluated using BaF-human Mpl, BaF-monkey Mpl, and BaF-mouse Mpl (Figures 9, 10, and 11).    AB317 had the highest agonistic activity against human, monkey, and mouse Mpl, whereas AB324 showed the highest agonistic activity against human and monkey Mpl.

[0159]

This proves that anti-Mpl diabodies having high agonistic activity can be obtained by the AGS method.

[Brief Description of the Drawings]

[0160]

[Fig. 1] Fig. 1 demonstrates the strategy for preparing single-chain antibody sc(Fv)$_2$.

[Fig. 2] Fig. 2 illustrates the assessment of VB22B sc(Fv)$_2$ binding activity using an Mpl-expressing CHO cell line.    Purified VB22B sc(Fv)$_2$ was used.

[Fig. 3] Fig. 3 illustrates the assessment of VB22B antibody agonistic activity using BaF-human Mpl.

[Fig. 4] Fig. 4 illustrates the assessment of VB22B antibody agonistic activity using BaF-monkey Mpl.

[Fig. 5] Fig. 5 illustrates the assessment of VB22B antibody agonistic activity using M-07e.

[Fig. 6] Fig. 6 shows the amino acid sequences of anti-human Mpl antibodies (H chains) that exhibit higher agonistic activities when converted into minibodies.

[Fig. 7] Fig. 7 shows the amino acid sequences of anti-human Mpl antibodies (L chains) which exhibit higher agonistic activities when converted into minibodies.

[Fig. 8] Fig. 8 illustrates the binding activity assessment of AB317 diabody using

Mpl-expressing CHO cells.    Both VB22B diabody (solid line) and AB317 diabody (broken line) were obtained from COS7 culture supernatants.

     [Fig. 9] Fig. 9 illustrates the agnostic activity assessment of AB324 and AB317 diabodies using BaF-human Mpl.

5        [Fig. 10] Fig. 10 illustrates the agnostic activity assessment of AB324 and AB317 diabodies using BaF-monkey Mpl.

     [Fig. 11] Fig. 11 illustrates the agnostic activity assessment of AB324 and AB317 diabodies using BaF-mouse Mpl.

[Sequence Listing]

SEQUENCE LISTING

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<213> Mus musculus

30

<400> 72
Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr
1               5                   10                  15


35

<210> 73

```
     <211>  7
     <212>  PRT
     <213>  Mus musculus


 5   <400>  73
     Arg Met Ser Asn Leu Ala Ser
     1               5


10   <210>  74
     <211>  9
     <212>  PRT
     <213>  Mus musculus


15   <400>  74
     Met Gln His Leu Glu Tyr Pro Tyr Thr
     1               5


20   <210>  75
     <211>  16
     <212>  PRT
     <213>  Mus musculus


25   <400>  75
     Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr
     1               5                   10                  15


30   <210>  76
     <211>  7
     <212>  PRT
     <213>  Mus musculus


35   <400>  76
     Arg Met Ser Asn Leu Ala Ser
```

1                5

<210> 77
5   <211> 9
<212> PRT
<213> Mus musculus

<400> 77
10  Met Gln His Leu Glu Tyr Pro Tyr Thr
1                5


<210> 78
15  <211> 16
<212> PRT
<213> Mus musculus

<400> 78
20  Arg Ser Ser Lys Ser Leu Leu Tyr Ser Asn Gly Asn Ile Tyr Leu Tyr
1                5                10                15


<210> 79
25  <211> 7
<212> PRT
<213> Mus musculus

<400> 79
30  Arg Met Ser Asn Leu Ala Ser
1                5


<210> 80
35  <211> 9
<212> PRT

<213> Mus musculus

<400> 80
Met Gln His Leu Glu Tyr Pro Tyr Thr
5    1              5


<210> 81
<211> 16
10   <212> PRT
<213> Mus musculus

<400> 81
Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr
15   1              5              10             15


<210> 82
<211> 7
20   <212> PRT
<213> Mus musculus

<400> 82
Arg Met Ser Asn Leu Ala Ser
25   1              5


<210> 83
<211> 9
30   <212> PRT
<213> Mus musculus

<400> 83
Met Gln His Leu Glu Tyr Pro Tyr Thr
35   1              5

<210> 84
<211> 16
<212> PRT
5   <213> Mus musculus


<400> 84
Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr
1               5                   10                  15
10


<210> 85
<211> 7
<212> PRT
15  <213> Mus musculus


<400> 85
Arg Met Ser Asn Leu Ala Ser
1               5
20


<210> 86
<211> 9
<212> PRT
25  <213> Mus musculus


<400> 86
Met Gln His Val Glu Tyr Pro Tyr Thr
1               5
30


<210> 87
<211> 16
<212> PRT
35  <213> Mus musculus

<400> 87

Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr
1               5                   10                  15

5

<210> 88
<211> 7
<212> PRT
<213> Mus musculus

10

<400> 88

Arg Met Ser Asn Leu Ala Ser
1               5

15

<210> 89
<211> 9
<212> PRT
<213> Mus musculus

20

<400> 89

Met Gln His Leu Glu Tyr Pro Tyr Thr
1               5

25

<210> 90
<211> 16
<212> PRT
<213> Mus musculus

30

<400> 90

Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr
1               5                   10                  15

35

<210> 91

<211> 7
<212> PRT
<213> Mus musculus

5    <400> 91
Arg Met Ser Asn Leu Ala Ser
1               5


10   <210> 92
<211> 9
<212> PRT
<213> Mus musculus

15   <400> 92
Met Gln His Leu Glu Tyr Pro Tyr Thr
1               5


20   <210> 93
<211> 16
<212> PRT
<213> Mus musculus

25   <400> 93
Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr
1               5                   10                  15


30   <210> 94
<211> 7
<212> PRT
<213> Mus musculus

35   <400> 94
Arg Met Ser Asn Leu Ala Ser

1                    5

<210> 95
<211> 9
<212> PRT
<213> Mus musculus

<400> 95
Met Gln His Ile Glu Tyr Pro Phe Thr
1               5

<210> 96
<211> 16
<212> PRT
<213> Mus musculus

<400> 96
Arg Ser Ser Lys Ser Leu Leu Tyr Ser Asn Gly Asn Thr Tyr Leu Tyr
1               5               10              15

<210> 97
<211> 7
<212> PRT
<213> Mus musculus

<400> 97
Arg Met Ser Asn Leu Ala Ser
1               5

<210> 98
<211> 9
<212> PRT

<213> Mus musculus

<400> 98
Met Gln His Leu Glu Tyr Pro Tyr Thr
5   1               5


<210> 99
<211> 16
10   <212> PRT
<213> Mus musculus

<400> 99
Arg Ser Ser Lys Ser Leu Leu Tyr Ser Asn Gly Asn Ile Tyr Leu Tyr
15   1               5                   10                  15


<210> 100
<211> 7
20   <212> PRT
<213> Mus musculus

<400> 100
Arg Met Ser Asn Leu Ala Ser
25   1               5


<210> 101
<211> 9
30   <212> PRT
<213> Mus musculus

<400> 101
Met Gln His Leu Glu Tyr Pro Tyr Thr
35   1               5

<210> 102
<211> 16
<212> PRT
5   <213> Mus musculus


<400> 102
Arg Ser Ser Lys Ser Leu Leu His Asn Asn Gly Asn Thr Tyr Leu Tyr
1                   5                   10                  15
10


<210> 103
<211> 7
<212> PRT
15  <213> Mus musculus


<400> 103
Arg Met Ser Asn Leu Ala Ser
1                   5
20


<210> 104
<211> 9
<212> PRT
25  <213> Mus musculus


<400> 104
Met Gln His Ile Glu Tyr Pro Phe Thr
1                   5
30


<210> 105
<211> 16
<212> PRT
35  <213> Mus musculus

<400> 105
Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr
1               5               10              15


5

<210> 106
<211> 7
<212> PRT
<213> Mus musculus

<400> 106
Arg Met Ser Asn Leu Ala Ser
1               5


15

<210> 107
<211> 9
<212> PRT
<213> Mus musculus

<400> 107
Met Gln His Leu Glu Tyr Pro Tyr Thr
1               5


25

<210> 108
<211> 15
<212> PRT
<213> Mus musculus

<400> 108
Arg Ala Ser Glu Ser Val Glu Tyr Tyr Gly Thr Ser Leu Met Gln
1               5               10              15


35

<210> 109

```
    <211>  7
    <212>  PRT
    <213>  Mus musculus


5   <400>  109
    Gly Ala Ser Asn Val Glu Ser
    1               5


10  <210>  110
    <211>  9
    <212>  PRT
    <213>  Mus musculus


15  <400>  110
    Gln Gln Ser Arg Lys Val Pro Trp Thr
    1               5


20  <210>  111
    <211>  11
    <212>  PRT
    <213>  Mus musculus


25  <400>  111
    Lys Ala Ser Gln Asn Val Gly Asn Ile Ile Ala
    1               5                   10


30  <210>  112
    <211>  7
    <212>  PRT
    <213>  Mus musculus


35  <400>  112
    Leu Ala Ser Tyr Arg Tyr Ser
```

1                    5


<210> 113
5    <211> 9
<212> PRT
<213> Mus musculus


<400> 113
10  Gln Gln Tyr Ser Ser Ser Pro Leu Thr
1                    5


<210> 114
15  <211> 12
<212> PRT
<213> Mus musculus


<400> 114
20  Ser Ala Ser Ser Ser Val Ser Ser Ser His Leu Tyr
1                    5                    10


<210> 115
25  <211> 7
<212> PRT
<213> Mus musculus


<400> 115
30  Ser Thr Ser Asn Leu Ala Ser
1                    5


<210> 116
35  <211> 9
<212> PRT

&lt;213&gt;  Mus musculus

&lt;400&gt;  116
His Gln Trp Ser Ser Tyr Pro Trp Thr
5    1                 5


&lt;210&gt;  117
&lt;211&gt;  411
10   &lt;212&gt;  DNA
&lt;213&gt;  Mus musculus

&lt;220&gt;
&lt;221&gt;  CDS
15   &lt;222&gt;  (1)..(411)
&lt;223&gt;


&lt;400&gt;  117
atg gaa tgg cct ttg atc ttt ctc ttc ctc ctg tca gga act gca ggt        48
20   Met Glu Trp Pro Leu Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1               5                   10                  15


gtc cac tcc cag gtt cag ctg cag cag tct gga cct gag ctg gtg aag        96
Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
25               20                  25                  30


cct ggg gcc tca gtg aag att tcc tgc aag gct tct ggc tat gca ttc       144
Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe
35                  40                  45
30
act aac tcc tgg atg aac tgg gtg aag cag agg cct gga aag ggt ctt       192
Thr Asn Ser Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu
50                  55                  60


35   gag tgg att gga cgg att tat cct gga gat gga gaa act atc tac aat       240
Glu Trp Ile Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn

65                    70                    75                    80

ggg aaa ttc agg gtc aag gcc aca ctg act gca gac aaa tcc tcc agc          288
Gly Lys Phe Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
                85                    90                    95

aca gcc tac atg gat atc agc agc ctg aca tct gag gac tct gcg gtc          336
Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
                100                   105                   110

tac ttc tgt gca aga ggc tat gat gat tac tcg ttt gct tac tgg ggc          384
Tyr Phe Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly
            115                   120                   125

caa ggg act ctg gtc act gtc tct gca                                       411
Gln Gly Thr Leu Val Thr Val Ser Ala
        130                   135


<210> 118
<211> 137
<212> PRT
<213> Mus musculus

<400> 118
Met Glu Trp Pro Leu Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1               5                   10                  15

Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
                20                  25                  30

Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe
            35                  40                  45

Thr Asn Ser Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu
        50                  55                  60

Glu Trp Ile Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn
65                    70                    75                    80

Gly Lys Phe Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
                    85                    90                    95

Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
                    100                    105                    110

Tyr Phe Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly
                    115                    120                    125

Gln Gly Thr Leu Val Thr Val Ser Ala
                    130                    135


<210> 119
<211> 396
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(396)
<223>

<400> 119
atg agg tgc cta gct gag ttc ctg ggg ctg ctt gtg ttc tgg att cct      48
Met Arg Cys Leu Ala Glu Phe Leu Gly Leu Leu Val Phe Trp Ile Pro
1                    5                    10                    15

gga gcc att ggg gat att gtg atg act cag gct gca ccc tct ata cct      96
Gly Ala Ile Gly Asp Ile Val Met Thr Gln Ala Ala Pro Ser Ile Pro
                    20                    25                    30

```
      gtc act cct gga gag tca gta tcc atc tcc tgt agg tct agt aag agt        144
      Val Thr Pro Gly Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser
              35                      40                  45
```

```
5     ctc ctg cat agt aat ggc aac act tac ttg tat tgg ttc ctg cag agg        192
      Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg
              50                      55                  60
```

```
      cca ggc cag tct cct caa ctc ctg ata tat cgg atg tcc aac ctt gcc        240
10    Pro Gly Gln Ser Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala
          65                  70                  75                  80
```

```
      tca gga gtc cca gat agg ttc agt ggc agt ggg tca gga act gct ttc        288
      Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe
15                      85                  90                  95
```

```
      aca ctg aga atc agt aga gtg gag gct gag gat gtg ggt gtt tat tac        336
      Thr Leu Arg Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr
                  100                 105                 110
20
      tgt atg caa cat ata gaa tat cct ttt acg ttc gga tcg ggg acc aag        384
      Cys Met Gln His Ile Glu Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys
                  115                 120                 125
```

```
25    ctg gaa ata aaa                                                         396
      Leu Glu Ile Lys
          130
```

```
30    <210>  120
      <211>  132
      <212>  PRT
      <213>  Mus musculus
```

```
35    <400>  120
      Met Arg Cys Leu Ala Glu Phe Leu Gly Leu Leu Val Phe Trp Ile Pro
```

```
       1              5                    10                   15

       Gly Ala Ile Gly Asp Ile Val Met Thr Gln Ala Ala Pro Ser Ile Pro
                  20                  25                  30
   5
       Val Thr Pro Gly Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser
                  35                  40                  45

       Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg
  10        50                  55                  60

       Pro Gly Gln Ser Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala
       65                  70                  75                  80

  15   Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe
                  85                  90                  95

       Thr Leu Arg Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr
                  100                 105                 110
  20
       Cys Met Gln His Ile Glu Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys
                  115                 120                 125

       Leu Glu Ile Lys
  25        130


       <210>  121
       <211>  762
  30   <212>  DNA
       <213>  Mus musculus

       <400>  121
       atggaatggc ctttgatctt tctcttcctc ctgtcaggaa ctgcaggtgt ccactcccag      60
  35
       gttcagctgc agcagtctgg acctgagctg gtgaagcctg gggcctcagt gaagatttcc     120
```

tgcaaggctt ctggctatgc attcactaac tcctggatga actgggtgaa gcagaggcct        180

ggaaagggtc ttgagtggat tggacggatt tatcctggag atggagaaac tatctacaat        240

gggaaattca gggtcaaggc cacactgact gcagacaaat cctccagcac agcctacatg        300

gatatcagca gcctgacatc tgaggactct gcggtctact tctgtgcaag aggctatgat        360

gattactcgt ttgcttactg gggccaaggg actctggtca ctgtctctgc aggtggtggt        420

ggttcggata ttgtgatgac tcaggctgca ccctctatac ctgtcactcc tggagagtca        480

gtatccatct cctgtaggtc tagtaagagt ctcctgcata gtaatggcaa cacttacttg        540

tattggttcc tgcagaggcc aggccagtct cctcaactcc tgatatatcg gatgtccaac        600

cttgcctcag gagtcccaga taggttcagt ggcagtgggt caggaactgc tttcacactg        660

agaatcagta gagtggaggc tgaggatgtg ggtgtttatt actgtatgca acatatagaa        720

tatccttttta cgttcggatc ggggaccaag ctggaaataa aa                          762


<210> 122
<211> 254
<212> PRT
<213> Mus musculus


<400> 122
Met Glu Trp Pro Leu Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1                5                  10                  15


Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
                20                  25                  30

```
      Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe
              35                  40                  45

      Thr Asn Ser Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu
  5       50                  55                  60

      Glu Trp Ile Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn
      65                  70                  75                  80

 10   Gly Lys Phe Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
                      85                  90                  95

      Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
                  100                 105                 110
 15
      Tyr Phe Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly
                  115                 120                 125

      Gln Gly Thr Leu Val Thr Val Ser Ala Gly Gly Gly Gly Ser Asp Ile
 20       130                 135                 140

      Val Met Thr Gln Ala Ala Pro Ser Ile Pro Val Thr Pro Gly Glu Ser
      145                 150                 155                 160

 25   Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly
                      165                 170                 175

      Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser Pro Gln
                  180                 185                 190
 30
      Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro Asp Arg
                  195                 200                 205

      Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile Ser Arg
 35       210                 215                 220
```

Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His Ile Glu
225                 230                 235                 240

Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
5                       245                 250


<210> 123
<211> 635
<212> PRT
<213> Homo sapiens


<400> 123
Met Pro Ser Trp Ala Leu Phe Met Val Thr Ser Cys Leu Leu Leu Ala
1               5                   10                  15

Pro Gln Asn Leu Ala Gln Val Ser Ser Gln Asp Val Ser Leu Leu Ala
                20                  25                  30

Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe Glu Asp Leu
            35                  40                  45

Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly Thr Tyr Gln
        50                  55                  60

Leu Leu Tyr Ala Tyr Pro Arg Glu Lys Pro Arg Ala Cys Pro Leu Ser
65                  70                  75                  80

Ser Gln Ser Met Pro His Phe Gly Thr Arg Tyr Val Cys Gln Phe Pro
                85                  90                  95

Asp Gln Glu Glu Val Arg Leu Phe Phe Pro Leu His Leu Trp Val Lys
                100                 105                 110

Asn Val Phe Leu Asn Gln Thr Arg Thr Gln Arg Val Leu Phe Val Asp
                115                 120                 125

Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala Met Gly Gly
    130                 135                 140

5   Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Glu Pro Ala Pro Glu
    145                 150                 155                 160

    Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro Arg Asp Pro
                    165                 170                 175

10
    Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala Thr Glu Thr
                    180                 185                 190

    Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala Leu Asp Gln
15              195                 200                 205

    Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly Pro Lys Gln
        210                 215                 220

20  Thr Ser Pro Ser Arg Glu Ala Ser Ala Leu Thr Ala Glu Gly Gly Ser
    225                 230                 235                 240

    Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp Leu Gln Leu
                    245                 250                 255

25
    Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp Gly Ser Trp
                    260                 265                 270

    Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val Ala Leu Gly
30              275                 280                 285

    Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys Gln Trp Gln
        290                 295                 300

35  Gln Gln Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His Ser Arg Ala
    305                 310                 315                 320

Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asn Cys Glu Glu
            325                 330                 335

5   Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe Ser Arg Cys
                340                 345                 350

    His Phe Lys Ser Arg Asn Asp Ser Ile Ile His Ile Leu Val Glu Val
            355                 360                 365

10
    Thr Thr Ala Pro Gly Thr Val His Ser Tyr Leu Gly Ser Pro Phe Trp
        370                 375                 380

    Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His Trp Arg Glu
15  385                 390                 395                 400

    Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro Ser Ser Trp
                405                 410                 415

20  Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly His
                420                 425                 430

    Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg Gly Gly Thr
                435                 440                 445

25
    Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu Arg Ala Arg
            450                 455                 460

    Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp Ser Asp Pro
30  465                 470                 475                 480

    Thr Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser Leu Val Thr
                485                 490                 495

35  Ala Leu His Leu Val Leu Gly Leu Ser Ala Val Leu Gly Leu Leu Leu
                500                 505                 510

Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu
        515                 520                 525

5  Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg
        530                 535                 540

Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser Asp Thr Cys
545                 550                 555                 560

10

Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu
                565                 570                 575

Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ala Gln Met Asp Tyr Arg
15              580                 585                 590

Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser Val Cys Pro
                595                 600                 605

20 Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile Ala Asn His
        610                 615                 620

Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro
625                 630                 635

25

<210>    124
<211>    122
<212>    PRT
30 <213>   Mus musculus

<400>    124
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1                   5                   10                  15

35

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser

                    20                    25                    30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
          35                    40                    45

Gly Arg Thr Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe
          50                    55                    60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
65                    70                    75                    80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                    85                    90                    95

Ala Arg Gly Trp Ile Leu Ala Asp Gly Gly Tyr Ser Phe Ala Tyr Trp
                    100                   105                   110

Gly Gln Gly Thr Leu Val Thr Val Ser Ala
          115                   120


<210> 125
<211> 112
<212> PRT
<213> Mus musculus


<400> 125
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Ile Pro Val Thr Pro Gly
1                 5                    10                    15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
          20                    25                    30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
          35                    40                    45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
    50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
5   65                  70                  75                  80

Ser Arg Val Glu Ala Glu Asp Val Gly Ile Tyr Tyr Cys Met Gln His
                    85                  90                  95

10  Leu Glu Tyr Pro Phe Thr Phe Gly Thr Gly Thr Lys Leu Glu Ile Lys
                        100                 105                 110


    <210>  126
15  <211>  118
    <212>  PRT
    <213>  Mus musculus

    <400>  126
20  Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
    1                   5                   10                  15

    Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser
                    20                  25                  30
25
    Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
                    35                  40                  45

    Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe
30      50                  55                  60

    Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
    65                  70                  75                  80

35  Ile Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                    85                  90                  95

Ala Arg Gly Tyr Ala Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
            100                 105             110

5   Leu Val Thr Val Ser Ala
            115


    <210>  127
10  <211>  112
    <212>  PRT
    <213>  Mus musculus


    <400>  127
15  Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
    1               5                   10              15

    Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
                20                  25              30
20
    Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
            35                  40                  45

    Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
25      50                  55                  60

    Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
    65                  70                  75                  80

30  Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85                  90                  95

    Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
                100                 105             110
35

<210> 128
<211> 118
<212> PRT
<213> Mus musculus

5

<400> 128
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1               5                   10                  15

10  Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser
                    20                  25                  30

    Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
                35                  40                  45

15

    Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Tyr Asn Gly Lys Phe
            50                  55                  60

    Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Asn Thr Ala Tyr
20  65                  70                  75                  80

    Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                    85                  90                  95

25  Ala Arg Gly Phe Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
                    100                 105                 110

    Leu Val Thr Val Ser Ala
                115

30

    <210> 129
    <211> 112
    <212> PRT
35  <213> Mus musculus

<400> 129

Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1               5                   10                  15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
                20                  25                  30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
            35                  40                  45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Ala Pro
        50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65                  70                  75                  80

Ser Arg Val Glu Thr Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85                  90                  95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

<210> 130
<211> 118
<212> PRT
<213> Mus musculus

<400> 130

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1               5                   10                  15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Ser Ser
                20                  25                  30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile

            35                    40                    45

        Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe
            50                    55                    60

5

        Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
        65                    70                    75                    80

        Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
10                        85                    90                    95

        Ala Ser Gly Tyr Ala Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
                    100                   105                   110

15      Leu Val Thr Val Ser Ala
                    115


        <210>   131
20      <211>   112
        <212>   PRT
        <213>   Mus musculus

        <400>   131
25      Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
        1                 5                     10                    15

        Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
                    20                    25                    30
30
        Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
                    35                    40                    45

        Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
35          50                    55                    60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65                  70                75                80


Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
5                   .85               90                95


Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
            100               105               110


10

<210> 132
<211> 118
<212> PRT
<213> Mus musculus

15

<400> 132
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1               5                 10                15


20    Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Arg Ser
                20                25                30


Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
          35                40                45

25

Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe
          50                55                60


Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
30    65                70                75                80


Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                90                95


35    Ala Ser Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
                100               105               110

Leu Val Thr Val Ser Ala
        115

5

<210> 133
<211> 112
<212> PRT
<213> Mus musculus

10

<400> 133
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1               5                   10                  15

15  Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
            20                  25                  30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
        35                  40                  45

20

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
        50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
25  65                  70                  75                  80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85                  90                  95

30  Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
                100                 105                 110

<210> 134
35  <211> 118
<212> PRT

<400> 134
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
5　1　　　　　　　5　　　　　　　　10　　　　　　　15

Ser Val Lys Ile Ser Cys Arg Ala Phe Gly Tyr Ala Phe Ser Asn Ser
　　　　　　20　　　　　　　25　　　　　　　30

10　Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
　　　　　　35　　　　　　　40　　　　　　　45

Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Asn Asn Gly Lys Phe
　　　　50　　　　　　　55　　　　　　　60
15

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
65　　　　　　　70　　　　　　　75　　　　　　　80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
20　　　　　　85　　　　　　　90　　　　　　　95

Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
　　　　　　100　　　　　　　105　　　　　　　110

25　Leu Val Thr Val Ser Ala
　　　　　　　115


<210> 135
30　<211> 112
<212> PRT
<213> Mus musculus

<400> 135
35　Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
　　1　　　　　　　5　　　　　　　10　　　　　　　15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
           20                 25               30

5   Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
           35                 40               45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
           50                 55               60

10

Asp Arg Phe Ser Gly Ser Gly Ser Gly Ala Ala Phe Thr Leu Arg Ile
65                 70               75               80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
15              85               90               95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
           100            105            110

20

<210> 136
<211> 115
<212> PRT
<213> Mus musculus

25

<400> 136
Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala
1            5               10               15

30   Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
           20                 25               30

Trp Val Asn Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile
           35                 40               45

35

Gly Arg Ile His Pro Ser Asp Ser Glu Thr His Cys Asn Gln Lys Phe

　　　　　　　50　　　　　　　　　　55　　　　　　　　　　60

　　　Lys Arg Lys Ala Thr Leu Thr Val Asn Lys Ser Ser Ser Thr Ala Tyr
　　　65　　　　　　　　　70　　　　　　　　　75　　　　　　　　　80

5

　　　Ile Gln Leu His Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
　　　　　　　　　　　85　　　　　　　　　90　　　　　　　　　95

10

　　　Thr Ser Gly Gly Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr
　　　　　　　　　100　　　　　　　　105　　　　　　　　110

　　　Val Ser Ala
　　　　　　　　115

15

　　　<210> 137
　　　<211> 112
　　　<212> PRT
　　　<213> Mus musculus

20

　　　<400> 137
　　　Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
　　　1　　　　　　　5　　　　　　　　　10　　　　　　　　15

25　Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu Tyr Ser
　　　　　　　　　20　　　　　　　　25　　　　　　　　30

　　　Asn Gly Asn Ile Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
　　　　　　　　　35　　　　　　　　40　　　　　　　　45

30

　　　Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
　　　　　　50　　　　　　　　55　　　　　　　　60

　　　Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
35　65　　　　　　　　70　　　　　　　　75　　　　　　　　80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85                  90                  95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
5               100             105                 110


<210> 138
<211> 118
10  <212> PRT
<213> Mus musculus


<400> 138
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
15  1               5               10                  15


Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser
                20                  25                  30


20  Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
                35                  40                  45


Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Asn Asn Gly Lys Phe
        50                  55                  60
25
Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Thr Thr Ala Tyr
65                  70                  75                  80


Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
30                  85                  90                  95


Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
                100             105                 110


35  Leu Val Thr Val Ser Ala
                115

<210> 139
<211> 112
5  <212> PRT
<213> Mus musculus

<400> 139
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
10 1               5                   10                  15

   Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
               20                  25                  30

15 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
               35                  40                  45

   Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
           50                  55                  60

20
   Asp Arg Phe Ser Gly Ser Gly Ser Gly Ala Ala Phe Thr Leu Arg Ile
   65                  70                  75                  80

   Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
25                   85                  90                  95

   Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
               100                 105                 110

30
   <210> 140
   <211> 118
   <212> PRT
   <213> Mus musculus
35
   <400> 140

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1               5                   10                  15

5      Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Thr Ser
                   20                  25                  30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
            35                  40                  45

10     Gly Arg Ile Tyr Pro Gly Asp Gly Glu Ala Asn Tyr Asn Gly Lys Phe
            50                  55                  60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Ser Ala Tyr
65                  70                  75                  80
15
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                  90                  95

Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
20                  100                 105                 110

Leu Val Thr Val Ser Ala
            115

25
       <210>  141
       <211>  112
       <212>  PRT
       <213>  Mus musculus
30
       <400>  141
       Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
       1               5                   10                  15

35     Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
                   20                  25                  30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Met Gln Arg Pro Gly Gln Ser
        35                  40                  45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
        50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65                  70                  75                  80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
            85                  90                  95

Val Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110


<210> 142
<211> 118
<212> PRT
<213> Mus musculus


<400> 142
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1               5               10                  15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser
            20                  25                  30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Pro Glu Trp Ile
            35                  40                  45

Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Tyr Asn Gly Lys Phe
        50                  55                  60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Val Tyr

65                    70                    75                    80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                    90                    95

Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
            100                   105                   110

Leu Val Thr Val Ser Ala
            115


<210> 143
<211> 112
<212> PRT
<213> Mus musculus

<400> 143
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1                 5                     10                    15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
            20                    25                    30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
            35                    40                    45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
        50                    55                    60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65                    70                    75                    80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85                    90                    95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110


5    <210>  144
     <211>  118
     <212>  PRT
     <213>  Mus musculus


10   <400>  144
     Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Leu Asn Pro Gly Ala
     1                 5                   10                  15


     Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Arg Ser
15               20                  25                  30


     Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
             35                  40                  45


20   Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Tyr Asn Gly Lys Phe
             50                  55                  60


     Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Thr Thr Ala Tyr
     65                  70                  75                  80
25
     Met Gln Phe Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                     85                  90                  95


     Ala Arg Gly Asp Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
30               100                 105                 110


     Leu Val Thr Val Ser Ala
                 115


35

     <210>  145

<211> 112
<212> PRT
<213> Mus musculus

5   <400> 145
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1               5                   10                  15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
10              20                  25                  30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
35                  40                  45

15  Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65              70                  75                  80
20
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
85                  90                  95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
25              100                 105                 110


<210> 146
<211> 115
30  <212> PRT
<213> Mus musculus

<400> 146
Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Arg Pro Gly Ala
35  1               5                   10                  15

Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr
            20              25                  30

Trp Val Asn Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile
5           35              40                  45

Gly Arg Ile His Pro Tyr Asp Ser Glu Thr His Tyr Asn Gln Lys Phe
        50              55                  60

10  Lys Asn Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
    65                  70                  75                  80

Ile Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

15
Ala Ser Gly Gly Trp Phe Ala Ser Trp Gly Gln Gly Thr Leu Val Thr
                100                 105                 110

Val Ser Ala
20          115


<210> 147
<211> 112
25  <212> PRT
<213> Mus musculus

<400> 147
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
30  1               5                   10                  15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu Tyr Ser
            20              25                  30

35  Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
            35              40                  45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
        50              55              60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Thr Ile
65              70              75              80

Ser Ser Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85              90              95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
            100             105             110

<210> 148
<211> 115
<212> PRT
<213> Mus musculus

<400> 148
Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala
1               5               10              15

Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr
            20              25              30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile
            35              40              45

Gly Arg Ile His Pro Phe Asp Ser Glu Thr His Cys Ser Gln Lys Phe
        50              55              60

Lys Asn Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr
65              70              75              80

Ile Gln Phe Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys

                    85                    90                    95

    Ser Ser Gly Gly Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr
                100                    105                    110

5

    Val Ser Ala
            115


10  <210>  149
    <211>  112
    <212>  PRT
    <213>  Mus musculus

15  <400>  149
    Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Ser Val Thr Pro Gly
    1               5               10                    15

    Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu Tyr Ser
20              20              25                    30

    Asn Gly Asn Ile Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
            35                    40                    45

25  Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
        50                    55                    60

    Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Lys Ile
    65              70                    75                    80

30
    Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85                    90                    95

    Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
35              100                    105                    110

&lt;210&gt;  150
&lt;211&gt;  118
&lt;212&gt;  PRT
5  &lt;213&gt;  Mus musculus


&lt;400&gt;  150
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1               5                   10                  15

10

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Ser
            20                  25                  30

Trp Met Asn Trp Val Arg Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
15          35                  40                  45

Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn Gly Lys Phe
        50                  55                  60

20  Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
    65                  70                  75                  80

Met Glu Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                  90                  95

25

Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
            100                 105                 110

Leu Val Thr Val Ser Ala
30          115


&lt;210&gt;  151
&lt;211&gt;  112
35  &lt;212&gt;  PRT
&lt;213&gt;  Mus musculus

<400> 151

Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1               5                   10                  15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Asn
            20                  25                  30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
            35                  40                  45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
        50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65                  70                  75                  80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85                  90                  95

Ile Glu Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110


<210> 152
<211> 118
<212> PRT
<213> Mus musculus

<400> 152

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1               5                   10                  15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Asn Ser
            20                  25                  30

```
        Trp Met Asn Trp Val Asn Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
                35                  40                  45


        Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Ile Tyr Asn Gly Asn Phe
   5        50                  55                  60


        Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Ile Ala Tyr
        65                  70                  75                  80


  10    Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                        85                  90                  95


        Thr Ser Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
                    100                 105                 110

  15
        Leu Val Thr Val Ser Ala
                    115



  20    <210>  153
        <211>  112
        <212>  PRT
        <213>  Mus musculus


  25    <400>  153
        Asp Ile Val Met Thr Gln Ala Ala Pro Ser Leu Pro Val Thr Pro Gly
        1               5                   10                  15


        Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
  30                20                  25                  30


        Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
                35                  40                  45


  35    Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
                50                  55                  60
```

```
     Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
     65              70                  75                  80


5    Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                     85                  90                  95


     Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
                     100             105             110
10


     <210>  154
     <211>  423
     <212>  DNA
15   <213>  Mus musculus


     <220>
     <221>  CDS
     <222>  (1)..(423)
20   <223>


     <400>  154
     atg gtt ctt gcc agc tct acc acc agc atc cac acc atg ctg ctc ctg      48
     Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu
25   1               5                   10                  15


     ctc ctg atg ctg gcc cag ccg gcc atg gcg gaa gtg aag ctg gtg gag      96
     Leu Leu Met Leu Ala Gln Pro Ala Met Ala Glu Val Lys Leu Val Glu
                     20                  25                  30
30
     tct ggg gga ggc tta gtg aag cct gga ggg tcc cgg aaa ctc tcc tgt     144
     Ser Gly Gly Gly Leu Val Lys Pro Gly Gly Ser Arg Lys Leu Ser Cys
                     35                  40                  45


35   gca gcc tct gga ttc act ttc agt agc tat acc atg tct tgg gtt cgc     192
     Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Thr Met Ser Trp Val Arg
```

```
            50                    55                    60

      cag act ccg gcg aag agg ctg gag tgg gtc gca acc att agt agt ggc      240
      Gln Thr Pro Ala Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Ser Gly
 5    65                    70                    75                    80


      agt agt acc atc tac tat gca gac aca gtg aag ggc cga ttc acc atc      288
      Ser Ser Thr Ile Tyr Tyr Ala Asp Thr Val Lys Gly Arg Phe Thr Ile
                        85                    90                    95
10
      tcc aga gac aat gcc aag aac acc ctg ttc ctg caa atg acc agt cta      336
      Ser Arg Asp Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Thr Ser Leu
                  100                   105                   110


15    agg tct gag gac aca gcc atg tat tac tgt gca agg aga tgg ttt ctt      384
      Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala Arg Arg Trp Phe Leu
                  115                   120                   125


      gac tgc tgg ggc caa ggc acc act ctc aca gtc tcc tcg                  423
20    Asp Cys Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
                  130                   135                   140



      <210>   155
25    <211>   141
      <212>   PRT
      <213>   Mus musculus


      <400>   155
30    Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu
      1                 5                   10                    15


      Leu Leu Met Leu Ala Gln Pro Ala Met Ala Glu Val Lys Leu Val Glu
                  20                    25                    30
35
      Ser Gly Gly Gly Leu Val Lys Pro Gly Gly Ser Arg Lys Leu Ser Cys
```

             35                40                45

        Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Thr Met Ser Trp Val Arg
            50                55                60

  5

        Gln Thr Pro Ala Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Ser Gly
        65                70                75                80

        Ser Ser Thr Ile Tyr Tyr Ala Asp Thr Val Lys Gly Arg Phe Thr Ile
 10                   85                90                95

        Ser Arg Asp Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Thr Ser Leu
                100                105                110

 15     Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala Arg Arg Trp Phe Leu
                115                120                125

        Asp Cys Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
                130                135                140

 20

        <210>   156
        <211>   357
        <212>   DNA
 25     <213>   Mus musculus

        <220>
        <221>   CDS
        <222>   (1)..(357)
 30     <223>

        <400>   156
        gat att gtg ctc acc caa tct cca gct tct ttg gct gtg tct cta ggg          48
        Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Ala Val Ser Leu Gly
 35     1                5                10                15

```
        cag agt gtc acc atc tcc tgc aga gcc agt gaa agt gtt gaa tat tat          96
        Gln Ser Val Thr Ile Ser Cys Arg Ala Ser Glu Ser Val Glu Tyr Tyr
                    20              25              30

 5      ggc act agt tta atg cag tgg tac caa cag aaa cca gga cag cca ccc          144
        Gly Thr Ser Leu Met Gln Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
                    35              40              45

        aaa ctc ctc atc tat ggt gca tcc aac gta gaa tct ggg gtc cct gcc          192
10      Lys Leu Leu Ile Tyr Gly Ala Ser Asn Val Glu Ser Gly Val Pro Ala
                    50              55              60

        agg ttt agt ggc agt ggg tct ggg aca gac ttc agc ctc aac atc cat          240
        Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Ser Leu Asn Ile His
15      65              70              75              80

        cct gtg gag gag gat gat att gca atg tat ttc tgt cag caa agt agg          288
        Pro Val Glu Glu Asp Asp Ile Ala Met Tyr Phe Cys Gln Gln Ser Arg
                        85              90              95
20
        aag gtt ccg tgg acg ttc ggt gga ggc acc aag ctg gaa ata aag gac          336
        Lys Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Asp
                        100             105             110

25      tac aag gat gac gac gat aag                                              357
        Tyr Lys Asp Asp Asp Asp Lys
                    115


30      <210>  157
        <211>  119
        <212>  PRT
        <213>  Mus musculus


35      <400>  157
        Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Ala Val Ser Leu Gly
```

```
       1              5                   10                   15

       Gln Ser Val Thr Ile Ser Cys Arg Ala Ser Glu Ser Val Glu Tyr Tyr
                   20                  25                  30

5

       Gly Thr Ser Leu Met Gln Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
                   35                  40                  45


       Lys Leu Leu Ile Tyr Gly Ala Ser Asn Val Glu Ser Gly Val Pro Ala
10         50                  55                  60


       Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Ser Leu Asn Ile His
       65                  70                  75                  80


15     Pro Val Glu Glu Asp Asp Ile Ala Met Tyr Phe Cys Gln Gln Ser Arg
                       85                  90                  95


       Lys Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Asp
                   100                 105                 110

20
       Tyr Lys Asp Asp Asp Asp Lys
                   115




25     <210>  158
       <211>  432
       <212>  DNA
       <213>  Mus musculus


30     <220>
       <221>  CDS
       <222>  (1)..(432)
       <223>


35     <400>  158
       atg gtt ctt gcc agc tct acc acc agc atc cac acc atg ctg ctc ctg        48
```

```
      Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu
      1               5                   10                  15


      ctc ctg atg ctg gcc cag ccg gcc atg gcg cag gtt cag ctc cag caa        96
  5   Leu Leu Met Leu Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Gln
                  20                  25                  30


      tct gga cct gag ctg gtg aag cct ggg gcc tca gtg aag att tcc tgc       144
      Ser Gly Pro Glu Leu Val Lys Pro Gly Ala Ser Val Lys Ile Ser Cys
 10           35                  40                  45


      aag gct tct ggc tat gca ttc agt agc tcc tgg atg aac tgg atg aag       192
      Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser Trp Met Asn Trp Met Lys
              50                  55                  60
 15
      cag agg cct gga aag ggt ctt gag tgg att ggg cgg att tat cct gga       240
      Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile Gly Arg Ile Tyr Pro Gly
      65                  70                  75                  80


 20   gat gga gat act aac tac aat ggg aag ttc aag ggc aag gcc aca ctg       288
      Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe Lys Gly Lys Ala Thr Leu
                      85                  90                  95


      act gca gac aaa tcc tcc agc aca gcc tac atg caa ctc agc agc ctg       336
 25   Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu
                  100                 105                 110


      aca tct gag gac tct gcg gtc tac ttc tgt gca aga gcg agg aaa act       384
      Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Ala Arg Ala Arg Lys Thr
 30               115                 120                 125


      tcc tgg ttt gct tac tgg ggc caa ggg act ctg gtc act gtc tct gcg       432
      Ser Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala
                  130                 135                 140
 35
```

```
<210>  159
<211>  144
<212>  PRT
<213>  Mus musculus

<400>  159
Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu
1               5                   10                  15


Leu Leu Met Leu Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Gln
            20                  25                  30


Ser Gly Pro Glu Leu Val Lys Pro Gly Ala Ser Val Lys Ile Ser Cys
        35                  40                  45


Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser Trp Met Asn Trp Met Lys
    50                  55                  60


Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile Gly Arg Ile Tyr Pro Gly
65                  70                  75                  80


Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe Lys Gly Lys Ala Thr Leu
                85                  90                  95


Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu
            100                 105                 110


Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Ala Arg Ala Arg Lys Thr
            115                 120                 125


Ser Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala
            130                 135                 140


<210>  160
<211>  345
```

<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(345)
<223>

<400> 160

```
gac att gtg ttg aca cag tct caa aaa ttc atg tcc aca tca gta gga        48
Asp Ile Val Leu Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
1               5                   10                  15


gac agg gtc agc atc agc tgc aag gcc agt cag aat gtg ggt aat att        96
Asp Arg Val Ser Ile Ser Cys Lys Ala Ser Gln Asn Val Gly Asn Ile
            20                  25                  30


ata gcc tgg tat caa cag aaa cca ggg caa tct cct aaa gca ctg att       144
Ile Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile
        35                  40                  45


tac ttg gca tcc tac cgg tac agt gga gtc cct gat cgc ttc aca ggc       192
Tyr Leu Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly
        50                  55                  60


agt gga tct ggg aca gat ttc act ctc acc att agt aat gtg cag tct       240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser
65                  70                  75                  80


gaa gac ttg gca gag tat ttc tgt cag caa tat agc agc tct ccg ctc       288
Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Ser Ser Ser Pro Leu
                85                  90                  95


acg ttc ggt gct ggg acc aag ctg gaa ata aag gac tac aag gat gac       336
Thr Phe Gly Ala Gly Thr Lys Leu Glu Ile Lys Asp Tyr Lys Asp Asp
                100                 105                 110
```

```
gac gat aag                                              345
Asp Asp Lys
        115
```

5

```
<210>  161
<211>  115
<212>  PRT
<213>  Mus musculus
```

10

```
<400>  161
Asp Ile Val Leu Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
1               5                   10                  15
```

15

```
Asp Arg Val Ser Ile Ser Cys Lys Ala Ser Gln Asn Val Gly Asn Ile
                20                  25                  30
```

```
Ile Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile
                35                  40                  45
```

20

```
Tyr Leu Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly
        50                  55                  60
```

```
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser
65                  70                  75                  80
```

25

```
Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Ser Ser Ser Pro Leu
                85                  90                  95
```

30

```
Thr Phe Gly Ala Gly Thr Lys Leu Glu Ile Lys Asp Tyr Lys Asp Asp
                100                 105                 110
```

```
Asp Asp Lys
        115
```

35

<210> 162
<211> 116
<212> PRT
5  <213> Mus musculus

<400> 162
Asp Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Gln
1               5                   10                  15

10

Ser Leu Ser Leu Thr Cys Thr Val Thr Gly Tyr Ser Ile Thr Ser Asp
            20                  25                  30

Tyr Ala Trp Ser Trp Ile Arg Gln Leu Pro Gly Asn Lys Leu Glu Trp
15          35                  40                  45

Met Gly Tyr Ile Thr Tyr Ser Gly Tyr Ser Ile Tyr Asn Pro Ser Leu
        50                  55                  60

20  Lys Ser Arg Ile Ser Ile Ser Arg Asp Thr Ser Lys Asn Gln Leu Phe
    65                  70                  75                  80

Leu Gln Leu Asn Ser Val Thr Thr Glu Asp Thr Ala Thr Tyr Tyr Cys
                85                  90                  95

25

Val Gly Gly Tyr Asp Asn Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
                100                 105                 110

Thr Val Ser Ser
30          115

<210> 163
<211> 108
35 <212> PRT
<213> Mus musculus

<400> 163

```
Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
1               5                   10                  15

Glu Lys Val Thr Leu Thr Cys Ser Ala Ser Ser Ser Val Ser Ser Ser
            20                  25                  30

His Leu Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Lys Leu Trp
            35                  40                  45

Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser
        50                  55                  60

Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Asn Met Glu
65                  70                  75                  80

Thr Glu Asp Ala Ala Ser Tyr Phe Cys His Gln Trp Ser Ser Tyr Pro
                85                  90                  95

Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
                100                 105
```

<210> 164
<211> 1924
<212> DNA
<213> Macaca fascicularis

<220>
<221> CDS
<222> (11)..(1918)
<223>

<400> 164

```
gaattccacc atg ccc tcc tgg gcc ctc ttc atg gtc acc tcc tgc ctc          49
```

```
                Met Pro Ser Trp Ala Leu Phe Met Val Thr Ser Cys Leu
                 1               5                   10


       ctc ctg gcc cct caa aac ctg gcc caa gtc agc agc caa gat gtc tcc      97
    5  Leu Leu Ala Pro Gln Asn Leu Ala Gln Val Ser Ser Gln Asp Val Ser
            15                  20                  25


       ttg ctg gcc tcg gac tca gag ccc ctg aag tgt ttc tcc cga aca ttt     145
       Leu Leu Ala Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe
   10  30                  35                  40                  45


       gag gac ctc act tgc ttc tgg gat gag gaa gag gca gca ccc agt ggg     193
       Glu Asp Leu Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly
                        50                  55                  60
   15
       aca tac cag ctg ctg tat gcc tac ccg ggg gag aag ccc cgt gcc tgc     241
       Thr Tyr Gln Leu Leu Tyr Ala Tyr Pro Gly Glu Lys Pro Arg Ala Cys
                    65                  70                  75


   20  ccc ctg agt tct cag agc gtg ccc cgc ttt gga acc cga tac gtg tgc     289
       Pro Leu Ser Ser Gln Ser Val Pro Arg Phe Gly Thr Arg Tyr Val Cys
                80                  85                  90


       cag ttt cca gcc cag gaa gaa gtg cgt ctc ttc tct ccg ctg cac ctc     337
   25  Gln Phe Pro Ala Gln Glu Glu Val Arg Leu Phe Ser Pro Leu His Leu
                95                  100                 105


       tgg gtg aag aat gtg ttc cta aac cag act cag att cag cga gtc ctc     385
       Trp Val Lys Asn Val Phe Leu Asn Gln Thr Gln Ile Gln Arg Val Leu
   30  110                 115                 120                 125


       ttt gtg gac agt gta ggc ctg ccg gct ccc ccc agt atc atc aag gcc     433
       Phe Val Asp Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala
                        130                 135                 140
   35
       atg ggt ggg agc cag cca ggg gaa ctt cag atc agc tgg gag gcc cca     481
```

```
          Met Gly Gly Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Ala Pro
                      145                 150                 155


          gct cca gaa atc agt gat ttc ctg agg tac gaa ctc cgc tat ggc ccc        529
     5    Ala Pro Glu Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro
                      160                 165                 170


          aaa gat ctc aag aac tcc act ggt ccc acg gtc ata cag ttg atc gcc        577
          Lys Asp Leu Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala
     10       175                 180                 185


          aca gaa acc tgc tgc cct gct ctg cag agg cca cac tca gcc tct gct        625
          Thr Glu Thr Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala
          190                 195                 200                 205
     15

          ctg gac cag tct cca tgt gct cag ccc aca atg ccc tgg caa gat gga        673
          Leu Asp Gln Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly
                          210                 215                 220


     20   cca aag cag acc tcc cca act aga gaa gct tca gct ctg aca gca gtg        721
          Pro Lys Gln Thr Ser Pro Thr Arg Glu Ala Ser Ala Leu Thr Ala Val
                      225                 230                 235


          ggt gga agc tgc ctc atc tca gga ctc cag cct ggc aac tcc tac tgg        769
     25   Gly Gly Ser Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp
                      240                 245                 250


          ctg cag ctg cgc agc gaa cct gat ggg atc tcc ctc ggt ggc tcc tgg        817
          Leu Gln Leu Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp
     30       255                 260                 265


          gga tcc tgg tcc ctc cct gtg act gtg gac ctg cct gga gat gca gtg        865
          Gly Ser Trp Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val
          270                 275                 280                 285
     35
          gca att gga ctg caa tgc ttt acc ttg gac ctg aag aat gtt acc tgt        913
```

```
     Ala Ile Gly Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys
                 290             295             300


     caa tgg cag caa gag gac cat gct agt tcc caa ggt ttc ttc tac cac      961
 5   Gln Trp Gln Gln Glu Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His
                 305             310             315


     agc agg gca cgg tgc tgc ccc aga gac agg tac ccc atc tgg gag gac     1009
     Ser Arg Ala Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asp
10               320             325             330


     tgt gaa gag gaa gag aaa aca aat cca gga tta cag acc cca cag ttc     1057
     Cys Glu Glu Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe
                 335             340             345
15
     tct cgc tgc cac ttc aag tca cga aat gac agc gtt att cac atc ctt     1105
     Ser Arg Cys His Phe Lys Ser Arg Asn Asp Ser Val Ile His Ile Leu
     350             355             360             365


20   gtg gag gtg acc aca gcc ctg ggt gct gtt cac agt tac ctg ggc tcc     1153
     Val Glu Val Thr Thr Ala Leu Gly Ala Val His Ser Tyr Leu Gly Ser
                 370             375             380


     cct ttc tgg atc cac cag gct gtg cgc ctc ccc acc cca aac ttg cac     1201
25   Pro Phe Trp Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His
                 385             390             395


     tgg agg gag atc tcc agc ggg cat ctg gaa ttg gag tgg cag cac cca     1249
     Trp Arg Glu Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro
30               400             405             410


     tca tcc tgg gca gcc caa gag acc tgc tat caa ctc cga tac aca gga     1297
     Ser Ser Trp Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly
         415             420             425
35
     gaa ggc cat cag gac tgg aag gtg ctg gag ccg cct ctc ggg gcc cga     1345
```

Glu Gly His Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg
430                435                440                445

    gga ggg acc ctg gag ctg cgc ccg cga tct cgc tac cgt tta cag ctg        1393
5   Gly Gly Thr Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu
                    450                455                460

    cgc gcc agg ctc aat ggc ccc acc tac caa ggt ccc tgg agc tcg tgg        1441
    Arg Ala Arg Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp
10                  465                470                475

    tcg gac cca gct agg gtg gag acc gcc acc gag acc gcc tgg att tcc        1489
    Ser Asp Pro Ala Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser
                    480                485                490
15
    ttg gtg acc gct ctg ctg cta gtg ctg ggc ctc agc gcc gtc ctg ggc        1537
    Leu Val Thr Ala Leu Leu Leu Val Leu Gly Leu Ser Ala Val Leu Gly
            495                500                505

20  ctg ctg ctg ctg agg tgg cag ttt cct gca cac tac agg aga ctg agg        1585
    Leu Leu Leu Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg
    510                515                520                525

    cat gcc ctg tgg ccc tca ctt cca gat ctg cac cga gtc cta ggc cag        1633
25  His Ala Leu Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln
                    530                535                540

    tac ctt agg gac act gca gcc ctg agt ccg ccc aag gcc aca gtc tca        1681
    Tyr Leu Arg Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser
30                  545                550                555

    gat acc tgt gaa gaa gtg gaa ccc agc ctc ctt gaa atc ctc ccc aag        1729
    Asp Thr Cys Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys
                    560                565                570
35
    tcc tca gag agg act cct ttg ccc ctg tgt tcc tcc cag tcc cag atg        1777

```
     Ser Ser Glu Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ser Gln Met
          575             580             585


     gac tac cga aga ttg cag cct tct tgc ctg ggg acc atg ccc ctg tct    1825
  5  Asp Tyr Arg Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser
     590             595             600             605


     gtg tgc cca ccc atg gct gag tca ggg tcc tgc tgt acc acc cac att    1873
     Val Cys Pro Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile
 10              610             615             620


     gcc aac cat tcc tac cta cca cta agc tat tgg cag cag cct tga       1918
     Ala Asn His Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro
               625             630             635

 15
     gtcgac                                                              1924
```

```
     <210>  165
 20  <211>  635
     <212>  PRT
     <213>  Macaca fascicularis


     <400>  165
 25  Met Pro Ser Trp Ala Leu Phe Met Val Thr Ser Cys Leu Leu Leu Ala
     1               5               10                  15


     Pro Gln Asn Leu Ala Gln Val Ser Ser Gln Asp Val Ser Leu Leu Ala
               20              25              30
 30
     Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe Glu Asp Leu
               35              40              45


     Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly Thr Tyr Gln
 35       50              55              60
```

Leu Leu Tyr Ala Tyr Pro Gly Glu Lys Pro Arg Ala Cys Pro Leu Ser
65              70              75              80

Ser Gln Ser Val Pro Arg Phe Gly Thr Arg Tyr Val Cys Gln Phe Pro
                85              90              95

Ala Gln Glu Glu Val Arg Leu Phe Ser Pro Leu His Leu Trp Val Lys
            100             105             110

Asn Val Phe Leu Asn Gln Thr Gln Ile Gln Arg Val Leu Phe Val Asp
            115             120             125

Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala Met Gly Gly
            130             135             140

Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Ala Pro Ala Pro Glu
145             150             155             160

Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro Lys Asp Leu
                165             170             175

Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala Thr Glu Thr
            180             185             190

Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala Leu Asp Gln
            195             200             205

Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly Pro Lys Gln
            210             215             220

Thr Ser Pro Thr Arg Glu Ala Ser Ala Leu Thr Ala Val Gly Gly Ser
225             230             235             240

Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp Leu Gln Leu
                245             250             255

Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp Gly Ser Trp
                260                  265              270

5    Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val Ala Ile Gly
              275              280              285

     Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys Gln Trp Gln
              290              295              300

10   Gln Glu Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His Ser Arg Ala
     305              310              315              320

     Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asp Cys Glu Glu
                   325              330              335
15
     Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe Ser Arg Cys
                   340              345              350

     His Phe Lys Ser Arg Asn Asp Ser Val Ile His Ile Leu Val Glu Val
20            355              360              365

     Thr Thr Ala Leu Gly Ala Val His Ser Tyr Leu Gly Ser Pro Phe Trp
              370              375              380

25   Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His Trp Arg Glu
     385              390              395              400

     Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro Ser Ser Trp
                   405              410              415
30
     Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly His
                   420              425              430

     Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg Gly Gly Thr
35            435              440              445

Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu Arg Ala Arg
   450                 455                 460

Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp Ser Asp Pro
5  465                 470                 475                 480

Ala Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser Leu Val Thr
                  485                 490                 495

10 Ala Leu Leu Leu Val Leu Gly Leu Ser Ala Val Leu Gly Leu Leu Leu
                  500                 505                 510

Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu
         515                 520                 525
15
Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg
         530                 535                 540

Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser Asp Thr Cys
20 545                 550                 555                 560

Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu
                  565                 570                 575

25 Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ser Gln Met Asp Tyr Arg
                  580                 585                 590

Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser Val Cys Pro
         595                 600                 605
30
Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile Ala Asn His
         610                 615                 620

Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro
35 625                 630                 635

<210> 166
<211> 24
<212> DNA
5  <213> Artificial


<220>
<223> an artificially synthesized sequence

10  <400> 166
cagggggccag tggatagact gatg                                    24



<210> 167
15  <211> 23
<212> DNA
<213> Artificial


<220>
20  <223> an artificially synthesized sequence


<400> 167
gctcactgga tggtgggaag atg                                      23


25

<210> 168
<211> 30
<212> DNA
<213> Artificial

30

<220>
<223> an artificially synthesized primer sequence


<400> 168
35  tagaattcca ccatggaatg gcctttgatc                               30

<210> 169
<211> 56
<212> DNA
5   <213> Artificial

<220>
<223> an artificially synthesized primer sequence

10  <400> 169
agcctgagtc atcacaatat ccgatccgcc tccacctgca gagacagtga ccagag        56

<210> 170
15  <211> 56
<212> DNA
<213> Artificial

<220>
20  <223> an artificially synthesized primer sequence

<400> 170
actctggtca ctgtctctgc aggtggaggc ggatcggata ttgtgatgac tcaggc        56

25

<210> 171
<211> 60
<212> DNA
<213> Artificial
30
<220>
<223> an artificially synthesized primer sequence

<400> 171
35  attgcggccg cttatcactt atcgtcgtca tccttgtagt cttttatttc cagcttggtc        60

<210> 172
<211> 8
<212> PRT
5  <213> Artificial


<220>
<223> an artificially synthesized FLAG tag sequence


10 <400> 172
Asp Tyr Lys Asp Asp Asp Asp Lys
1                 5



15 <210> 173
<211> 85
<212> DNA
<213> Artificial


20 <220>
<223> an artificially synthesized primer sequence


<400> 173
tagaattcca ccatggaatg gcctttgatc tttctcttcc tcctgtcagg aactgcaggt       60
25
gtccactccc aggttcagct gcagc                                            85



<210> 174
30 <211> 82
<212> DNA
<213> Artificial


<220>
35 <223> an artificially synthesized primer sequence

<400> 174
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cggatattgt gatgactcag gc                                              82

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cctgcagaga cagtgaccag ag                                              82

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cttttatttc cagcttggtc c                                                  81

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gcccagccgg ccatggcgca ggtycarctg cagcagtc                                    38

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&lt;211&gt;  38
&lt;212&gt;  DNA
&lt;213&gt;  Artificial
15
&lt;220&gt;
&lt;223&gt;  an artificially synthesized primer sequence

&lt;400&gt;  184
20  gcccagccgg ccatggcgca ggtccacgtg aagcagtc                                    38

&lt;210&gt;  185
&lt;211&gt;  38
25  &lt;212&gt;  DNA
&lt;213&gt;  Artificial

&lt;220&gt;
&lt;223&gt;  an artificially synthesized primer sequence
30
&lt;400&gt;  185
gcccagccgg ccatggcgga ggtgaasstg gtggaatc                                    38

35  &lt;210&gt;  186
&lt;211&gt;  38

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      <220>
 5    <223>  an artificially synthesized primer sequence

      <400>  186
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      <211>  38
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      <213>  Artificial

15

      <220>
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      <400>  187
20    gcccagccgg ccatggcgga ggtgcagskg gtggagtc                              38



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25    <212>  DNA
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      <400>  188
      gcccagccgg ccatggcgga kgtgcamctg gtggagtc                              38



35    <210>  189
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      <213>  Artificial

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      <210>  194
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      <213>  Artificial

      <220>
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35    <210>  195
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10

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15

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20  gcccagccgg ccatggcgga ggtgaaggtc atcgagtc                            38



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     <220>
5    <223>  an artificially synthesized primer sequence


     <400>  198
     ggagccgccg ccgcccgagg agactgtgag agtggt                              36


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     <210>  199
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     <213>  Artificial
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     <220>
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     <400>  199
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     <210>  200
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25   <212>  DNA
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     <400>  200
     ggagccgccg ccgcccgagg agacggtgac tgaggt                              36



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<212> DNA
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ggcggcggcg gctccgayat tcagatgayd cagtc                                35


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&lt;211&gt;  35
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&lt;213&gt;  Artificial
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&lt;223&gt;  an artificially synthesized primer sequence

&lt;400&gt;  208
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&lt;210&gt;  209
&lt;211&gt;  35
25  &lt;212&gt;  DNA
&lt;213&gt;  Artificial

&lt;220&gt;
&lt;223&gt;  an artificially synthesized primer sequence
30
&lt;400&gt;  209
ggcggcggcg gctccgayat tgttctcawc cagtc                                35


35  &lt;210&gt;  210
&lt;211&gt;  35

&lt;212&gt;  DNA
&lt;213&gt;  Artificial


&lt;220&gt;
5   &lt;223&gt;  an artificially synthesized primer sequence


&lt;400&gt;  210
ggcggcggcg gctccgayat tgwgctsacc caatc                                    35


10


&lt;210&gt;  211
&lt;211&gt;  35
&lt;212&gt;  DNA
&lt;213&gt;  Artificial

15


&lt;220&gt;
&lt;223&gt;  an artificially synthesized primer sequence


&lt;400&gt;  211
20  ggcggcggcg gctccgayat tstratgacc cartc                                    35



&lt;210&gt;  212
&lt;211&gt;  35
25  &lt;212&gt;  DNA
&lt;213&gt;  Artificial


&lt;220&gt;
&lt;223&gt;  an artificially synthesized primer sequence

30

&lt;400&gt;  212
ggcggcggcg gctccgayrt tktgatgacc carac                                    35



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&lt;211&gt;  35

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&lt;220&gt;
5  &lt;223&gt;  an artificially synthesized primer sequence


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10

&lt;210&gt;  217
&lt;211&gt;  35
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15

&lt;220&gt;
&lt;223&gt;  an artificially synthesized primer sequence


&lt;400&gt;  217
20  ggcggcggcg gctccgayat tttgctgact cagtc                    35



&lt;210&gt;  218
&lt;211&gt;  38
25  &lt;212&gt;  DNA
&lt;213&gt;  Artificial


&lt;220&gt;
&lt;223&gt;  an artificially synthesized primer sequence
30

&lt;400&gt;  218
ggcggcggcg gctccgatgc tgttgtgact caggaatc                    38



35  &lt;210&gt;  219
&lt;211&gt;  36

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<400>  219
ggaattcggc ccccgaggcc ttgatttcca gcttgg                    36


<210>  220
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ggaattcggc ccccgaggcc tttatttcca gcttgg                    36


<210>  221
<211>  36
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ggaattcggc ccccgaggcc tttatttcca actttg                    36


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<211>  36
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<212> DNA
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5   <223>  an artificially synthesized primer sequence


<400>  222
ggaattcggc ccccgaggcc ttcagctcca gcttgg                        36


10


<210>  223
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15

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<400>  223
20  ggaattcggc ccccgaggcc cctaggacag tcagtttgg                   39



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25  <212>  DNA
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ttactcgcgg cccagccggc catggcg                                  27



35  <210>  225
<211>  17

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    <220>
5   <223> an artificially synthesized sequence

    <400> 225
    ggaattcggc ccccgag                                    17


10

    <210> 226
    <211> 20
    <212> DNA
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    <220>
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    <400> 226
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    <210> 227
    <211> 20
25  <212> DNA
    <213> Artificial

    <220>
    <223> an artificially synthesized sequence
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    <400> 227
    caggtggggt ctttcattcc                                 20

[Document Name] Drawings
[Fig. 1]

[Fig. 2]



**CHO-HUMAN Mpl**

FITC LOG

**CHO-MONKEY Mpl**

FITC LOG

**CHO-MOUSE Mpl**

FITC LOG

**CHO**

FITC LOG
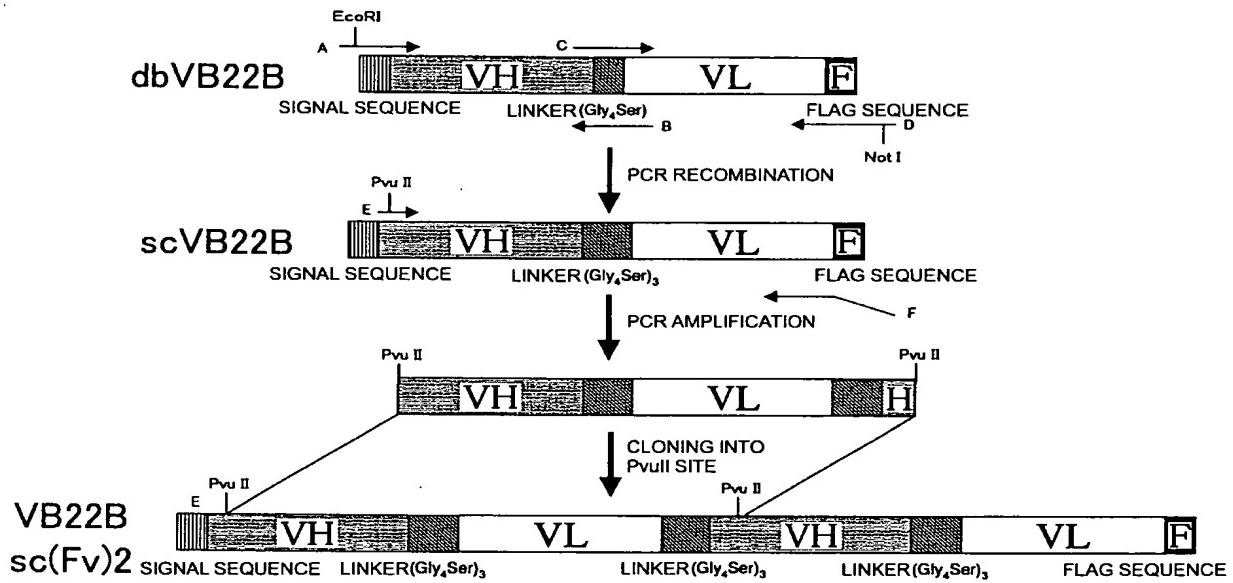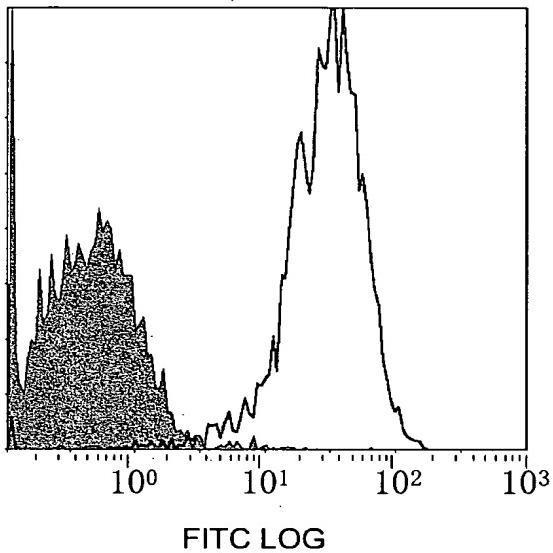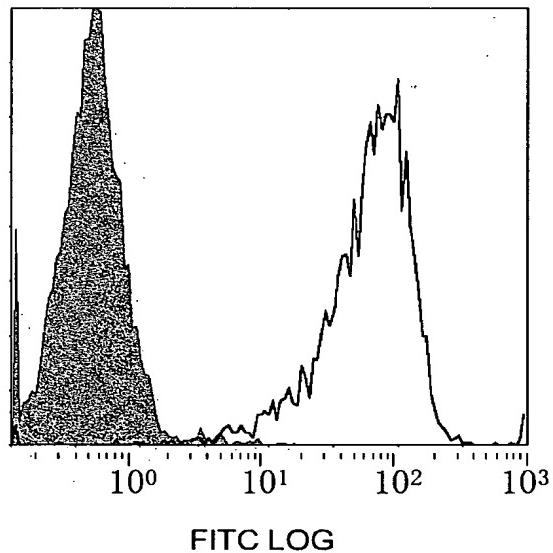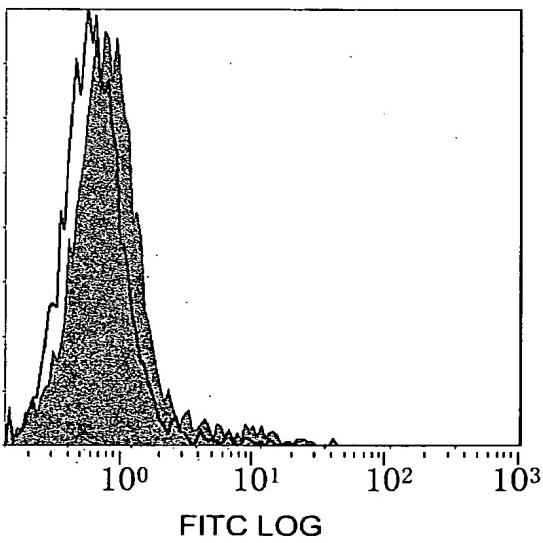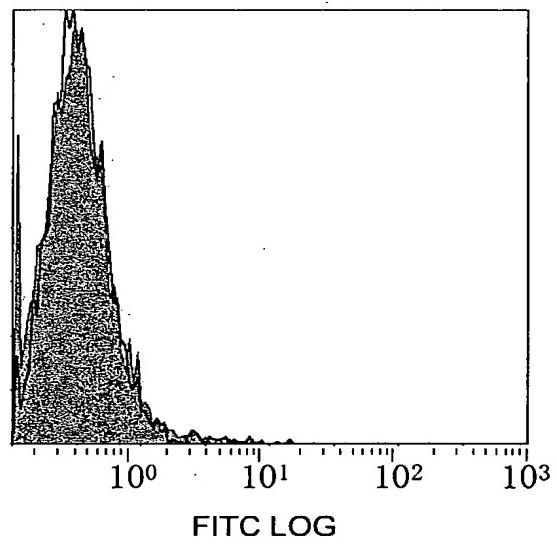
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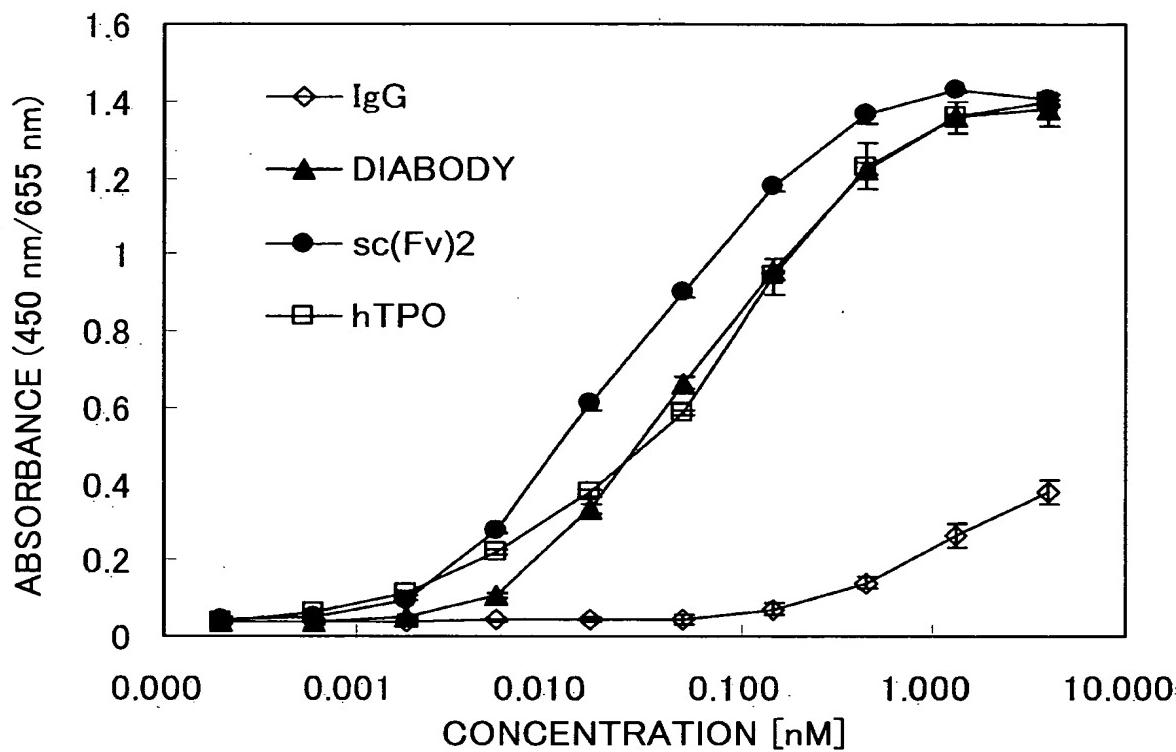


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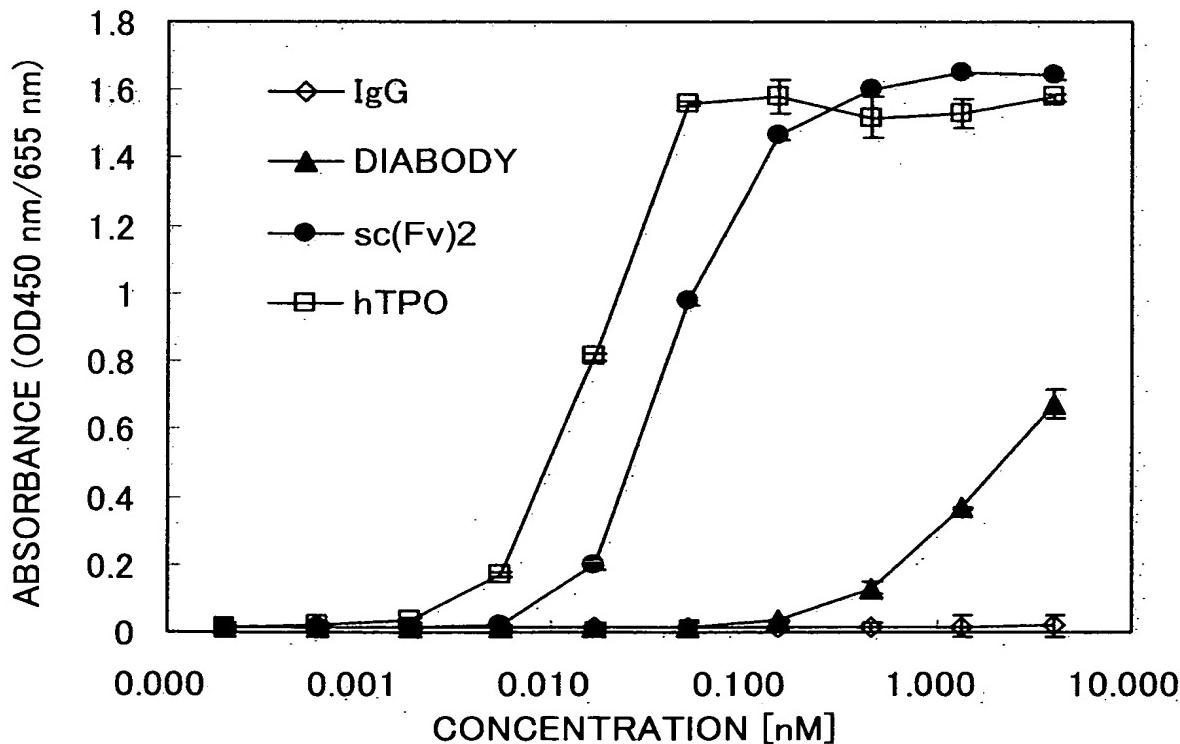
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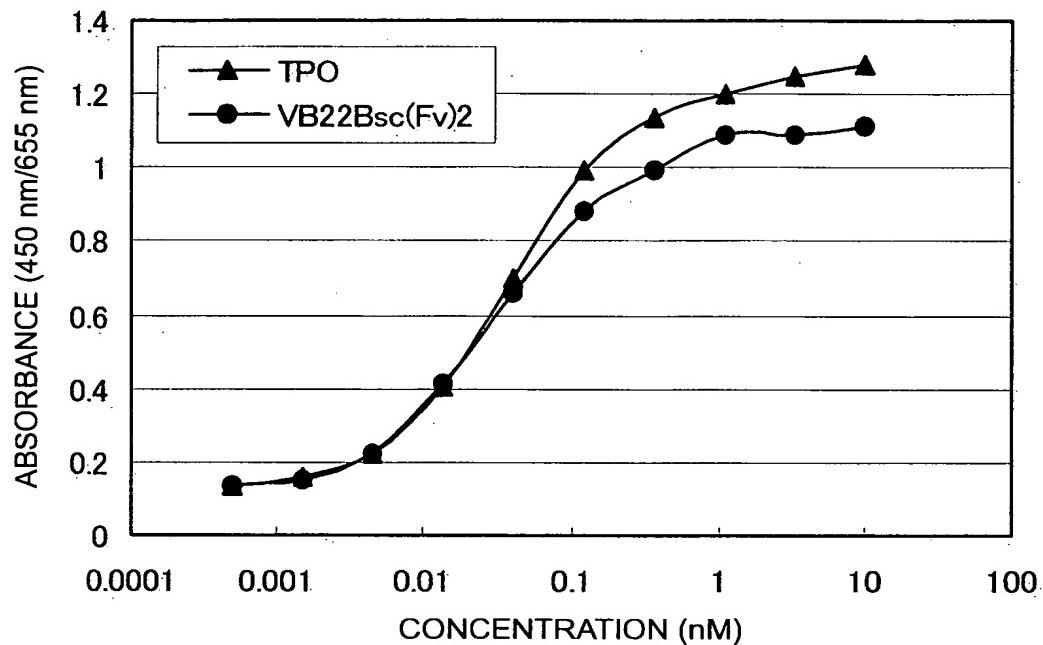


[Fig. 6]



|  |  | CDR1 |  | CDR2 |
|---|---|---|---|---|
| VA7 | QVQLQQSGPELVKPGASVKISCKASGYAFS | SSWMN | WVKQRPGKGLEWIG | RTYPGDGDTNYNGKFKG |
| VA130 | QVQLQQSGPELVKPGASVKISCKASGYAFS | SSWMN | WVKQRPGKGLEWIG | RIYPGDGDTNYNGKFKG |
| VA259 | QVQLQQSGPELVKPGASVKISCKASGYAFS | SSWMN | WVKQRPGKGLEWIG | RIYPGDGETNYNGKFKG |
| VB17B | QVQLQQSGPELVKPGASVKISCKASGYTFS | SSWMN | WVKQRPGKGLEWIG | RIYPGDGDTNYNGKFKG |
| VB12B | QVQLQQSGPELVKPGASVKISCKASGYAFS | RSWMN | WVKQRPGKGLEWIG | RIYPGDGDTNYNGKFKG |
| VB140 | QVQLQQSGPELVKPGASVKISCRAFGYAFS | NSWMN | WVKQRPGKGLEWIG | RIYPGDGETNNNGKFKG |
| VB33 | QVQLQQPGAELVKPGASVKLSCKASGYTFT | NYWVN | WVKQRPGRGLEWIG | RIHPSDSETHCNQKFKR |
| VB45B | QVQLQQPGPELVKPGASVKISCKASGYAFS | SSWMN | WVKQRPGKGLEWIG | RIYPGDGETNNNGKFKG |
| VB8B | QVQLQQSGPELVKPGASVKISCKASGYAFS | TSWMN | WVKQRPGKGLEWIG | RIYPGDGEANYNGKFKG |
| VB115 | QVQLQQSGPELVKPGASVKISCKASGYAFS | SSWMN | WVKQRPGKGPEWIG | RIYPGDGETNYNGKFKG |
| VB14B | QVQLQQSGPELLNPGASVKISCKASGYAFS | RSWMN | WVKQRPGKGLEWIG | RIYPGDGETNYNGKFKG |
| VB22B | QVQLQQSGPELVKPGASVKISCKASGYAFT | NSWMN | WVKQRPGKGLEWIG | RIYPGDGETIYNGKFRV |
| VB16 | QVQLQQPGTELVRPGASVKLSCKASGYTFT | DYWVN | WVKQRPGRGLEWIG | RIHPYDSETHYNQKFKN |
| VB157 | QVQLQQPGAELVKPGASVKLSCKASGYTFT | DYWMN | WVKQRPGRGLEWIG | RIHPFDSETHCSQKFKN |
| VB4B | QVQLQQSGPELVKPGASVKISCKASGYAFT | NSWMN | WVRQRPGKGLEWIG | RIYPGDGETIYNGKFRV |
| VB51 | QVQLQQSGPELVKPGASVKISCKASGYAFS | NSWMN | WVNQRPGKGLEWIG | RIYPGDGDTIYNGNFKG |

|  |  | CDR3 |  |
|---|---|---|---|
| VA7 | KATLTADKSSSTAYMQLSSLTSEDSAVYFCAR | GWILADGGYSFAY | WGQGTLVTVSA |
| VA130 | KATLTADKSSSTAYIQLSSLTSEDSAVYFCAR | GYAD----YSFAY | WGQGTLVTVSA |
| VA259 | KATLTADKSSNTAYMQLSSLTSEDSAVYFCAR | GFGD----YSFAY | WGQGTLVTVSA |
| VB17B | KATLTADKSSSTAYMQLSSLTSEDSAVYFCAS | GYAD----YSFAY | WGQGTLVTVSA |
| VB12B | KATLTADKSSSTAYMQLSSLTSEDSAVYFCAS | GYDD----YSFAY | WGQGTLVTVSA |
| VB140 | KATLTADKSSSTAYMQLSSLTSEDSAVYFCAR | GYGD----YSFAY | WGQGTLVTVSA |
| VB33 | KATLTVNKSSSTAYIQLHSLTSEDSAVYYCTS | GGW------FAY | WGQGTLVTVSA |
| VB45B | KATLTADKSSTTAYMQLSSLTSEDSAVYFCAR | GYGD----YSFAY | WGQGTLVTVSA |
| VB8B | KATLTADKSSSSAYMQLSSLTSEDSAVYFCAR | GYGD----YSFAY | WGQGTLVTVSA |
| VB115 | KATLTADKSSSTVYMQLSSLTSEDSAVYFCAR | GYGD----YSFAY | WGQGTLVTVSA |
| VB14B | KATLTADKSSTTAYMQFSSLTSEDSAVYFCAR | GDGD----YSFAY | WGQGTLVTVSA |
| VB22B | KATLTADKSSSTAYMDISSLTSEDSAVYFCAR | GYDD----YSFAY | WGQGTLVTVSA |
| VB16 | KATLTVDKSSSTAYIQLSSLTSEDSAVYYCAS | GGW------FAS | WGQGTLVTVSA |
| VB157 | KATLTVDKSSNTAYIQFSSLTSEDSAVYYCSS | GGW------FAY | WGQGTLVTVSA |
| VB4B | KATLTADKSSSTAYMEISSLTSEDSAVYFCAR | GYDD----YSFAY | WGQGTLVTVSA |
| VB51 | KATLTADKSSSIAYMQLSSLTSEDSAVYFCTS | GYDD----YSFAY | WGQGTLVTVSA |

[Fig. 7]

| | | CDR1 | | CDR2 |
|---|---|---|---|---|
| VA7 | DIVMTQAAPSIPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VA130 | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VA259 | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB17B | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB12B | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB140 | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB33 | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLYSNGNIYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB45B | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB8B | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFMQRPGQSPQLLIY | RMSNLAS |
| VB115 | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB14B | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB22B | DIVMTQAAPSIPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB16 | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLYSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB157 | DIVMTQAAPSVSVTPGESVSISC | RSSKSLLYSNGNIYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB4B | DIVMTQAAPSVPVTPGESVSISC | RSSKSLLHNNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |
| VB51 | DIVMTQAAPSLPVTPGESVSISC | RSSKSLLHSNGNTYLY | WFLQRPGQSPQLLIY | RMSNLAS |

| | | CDR3 | |
|---|---|---|---|
| VA7 | GVPDRFSGSGSGTAFTLRISRVEAEDVGIYYC | MQHLEYPFT | FGTGTKLEIK |
| VA130 | GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VA259 | GAPDRFSGSGSGTAFTLRISRVETEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VB17B | GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VB12B | GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VB140 | GVPDRFSGSGSGAAFTLRISRVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VB33 | GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VB45B | GVPDRFSGSGSGAAFTLRISRVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VB8B | GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC | MQHVEYPYT | FGSGTKLEIK |
| VB115 | GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VB14B | GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VB22B | GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC | MQHIEYPFT | FGSGTKLEIK |
| VB16 | GVPDRFSGSGSGTAFTLTISSVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VB157 | GVPDRFSGSGSGTAFTLKISRVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |
| VB4B | GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC | MQHIEYPFT | FGSGTKLEIK |
| VB51 | GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC | MQHLEYPYT | FGSGTKLEIK |

[Fig. 8]

## CHO-HUMAN Mpl

FITC LOG

## CHO-MONKEY Mpl

FITC LOG

## CHO-MOUSE Mpl

FITC LOG

## CHO

FITC LOG

[Fig. 9]



[Fig. 10]



5
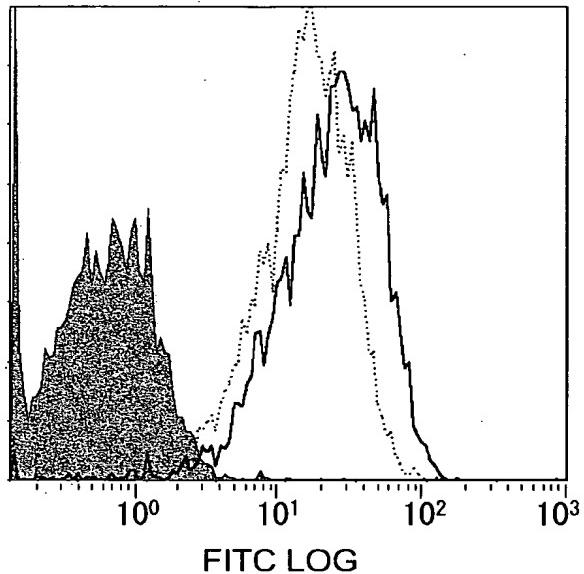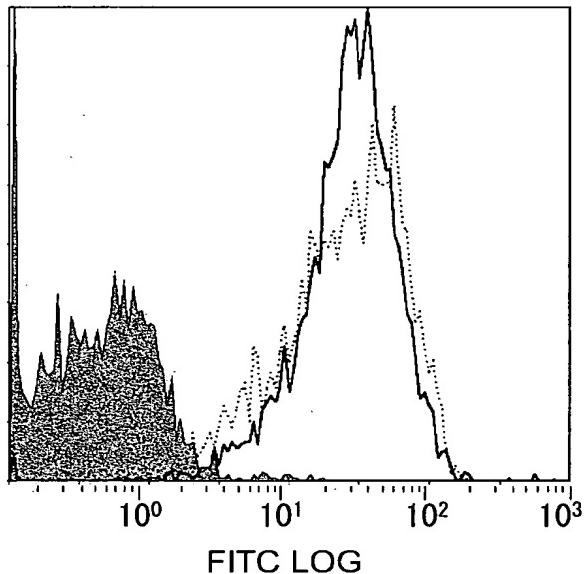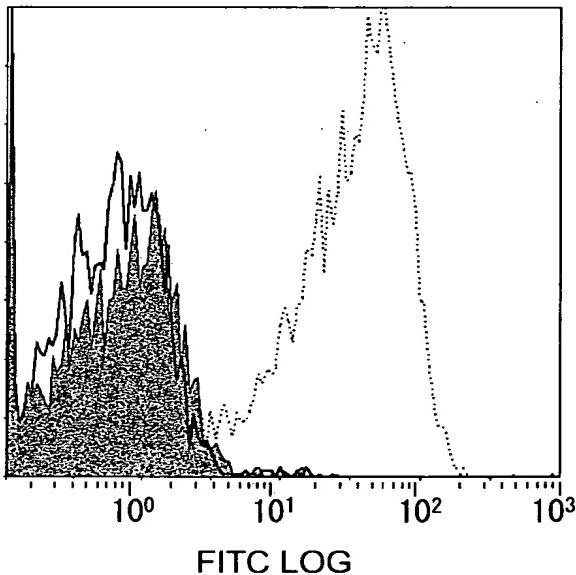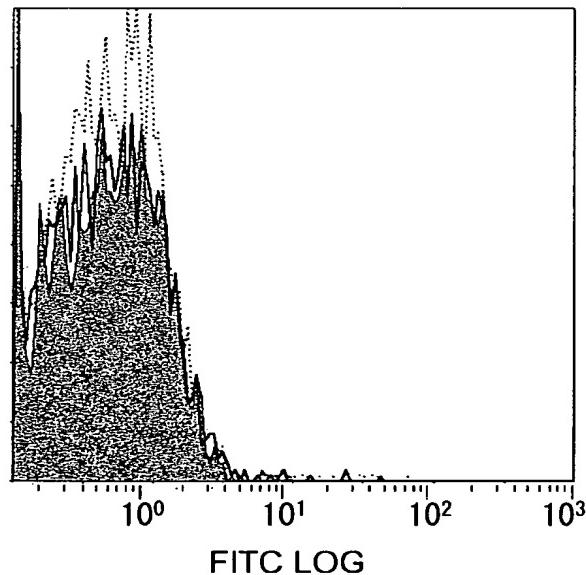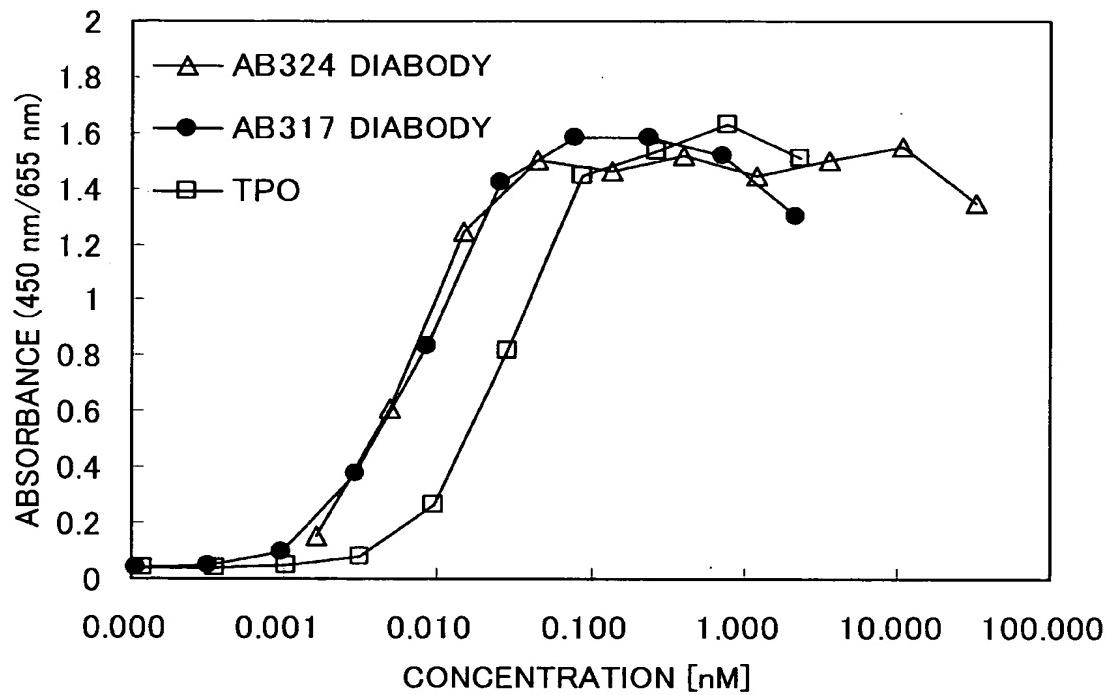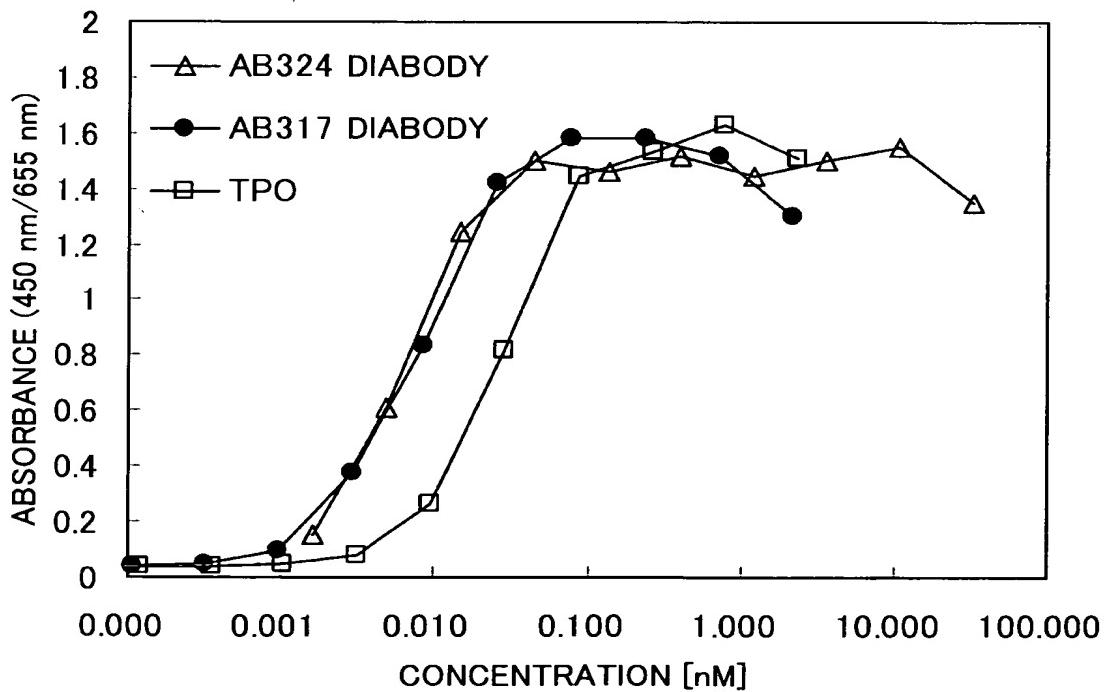
[Fig. 11]
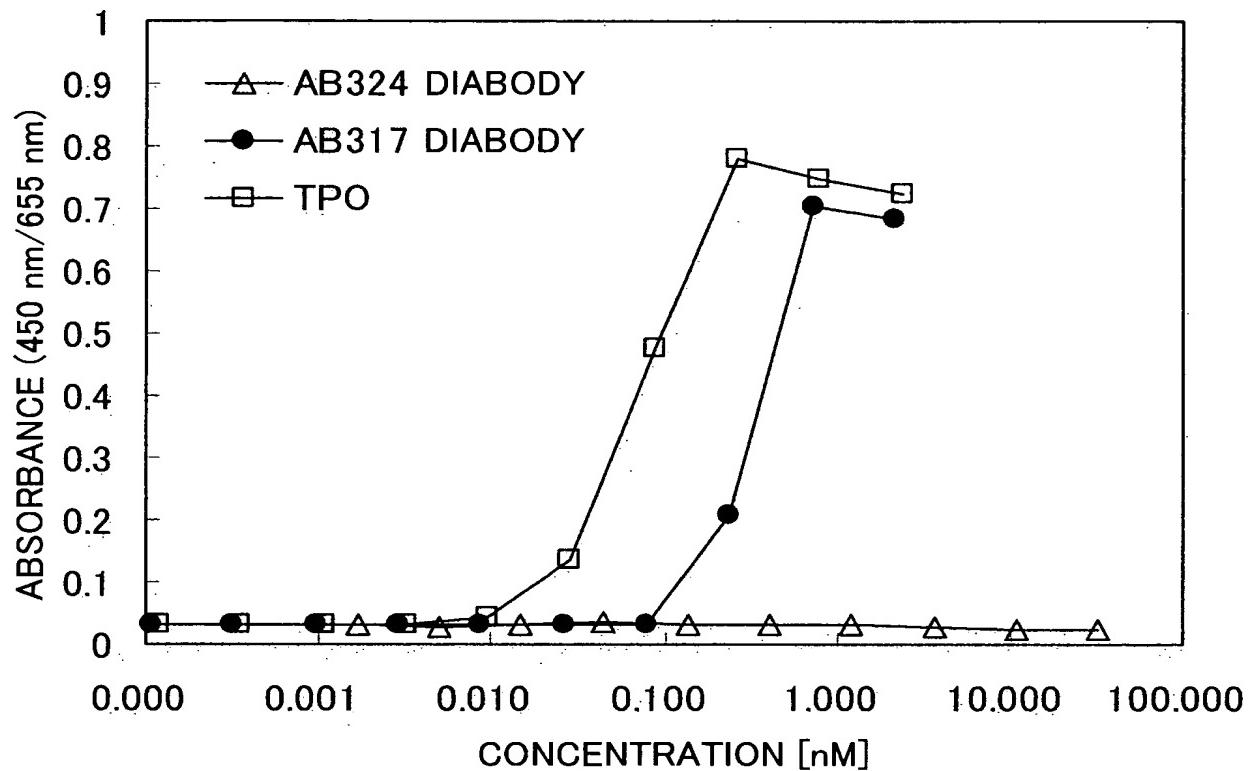
[Document Name] Abstract

[Abstract]

[Problems to be Solved] An objective of the present invention is to provide novel anti-Mpl antibodies having TPO-agonistic activity.

[Means for Solving the Problems] Anti-human Mpl antibodies were isolated and purified, and then anti-human Mpl diabodies and anti-human Mpl sv(Fv)$_2$ were purified using genetic engineering techniques.   Furthermore, the present inventors succeeded in humanizing anti-human Mpl sc(Fv)$_2$.

The diabodies and sc(Fv)$_2$ were assayed for TPO-like agonistic activity, and were found to have activities higher than those of anti-human Mpl antibodies, or activities equivalent to or higher than those of naturally-occurring human TPO ligand.

[Selected Drawings] None